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(51) International Patent Classification ⁷ : A01N 43/04, C08B 37/08	A1	(11) International Publication Number: WO 00/36918 (43) International Publication Date: 29 June 2000 (29.06.00)
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(54) Title: METHODS AND COMPOSITIONS FOR TREATMENT OF CELL PROLIFERATIVE DISORDERS (57) Abstract <p>The present invention relates to methods and compositions comprising at least one endothelin antagonist, preferably in combination with a poly-β-1\rightarrow-4-N-acetylglucosamine (p-GlcNAc) polysaccharide matrix, for use in the treatment of cancer and other proliferative diseases. The endothelin antagonist can be a peptide or non-peptide compound, and the p-GlcNAc matrix of the invention is comprised of a polymer of high molecular weight whose constituent monosaccharide sugars are attached in a β-1\rightarrow-4 conformation, and which is free of proteins, and substantially free of single amino acids, and other organic and inorganic contaminants. The compositions and methods of the invention are useful for inhibiting the growth of tumors and other neoplastic cells and/or for inhibiting the metastasis of neoplastic cells <i>in vivo</i>.</p>		

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METHODS AND COMPOSITIONS FOR TREATMENT OF
CELL PROLIFERATIVE DISORDERS

The present application is a continuation-in-part of United States application Ser. No. 09/218,288, filed December 22, 1998, which is a continuation-in-part of United States application Ser. No. 471,290, filed June 6, 1995, now United States Patent 5,858,350, which application is a continuation-in-part of United States application Ser. No. 347,911, filed December 1, 1994, now United States Patent 5,623,064, which is a continuation-in-part of United States application, Ser. No. 160,569, filed December 1, 1993, now United States Patent 5,622,834, which applications are incorporated herein by reference in their entireties.

1. INTRODUCTION

The present invention relates to methods and compositions comprising at least one endothelin antagonist, preferably in combination with a poly- β -1-4-N-acetylglucosamine (p-GlcNAc) polysaccharide matrix, for use in the treatment of cancer and other proliferative diseases. More specifically, the endothelin antagonist of the invention can be a peptide or non-peptide compound, and the p-GlcNAc matrix of the invention is comprised of a polymer of high molecular weight whose constituent monosaccharide sugars are attached in a β -1-4 conformation, and which is free of proteins, and substantially free of single amino acids, and other organic and inorganic contaminants. The compositions and methods of the invention are useful for inhibiting the growth of tumors and other neoplastic cells and/or for inhibiting the metastasis of neoplastic cells in vivo.

2. BACKGROUND OF THE INVENTION

The endothelins are a family of 21-amino acid peptides, e.g., ET-1, ET-2, and ET-3, originally characterized by their potent vasoconstricting and angiogenic properties (see, e.g., Luscher et al., 1995, Agents Actions Suppl. (Switzerland) 45: 237-253; Yanagisawa et al., 1988, Nature 332: 411-415).

These peptides additionally appear to be related to growth factors such as bFGF and often act in synergy with them (see, e.g., Halaban, 1996, Seminars in Oncology 23: 673-681; Reid et al., 1996, Development 122: 3911-3919; Markewitz et al., 5 1995, Am. J. Physiol. 268: L192-L200; and Nelson et al., 1996, Cancer Res. 56: 663-668). Furthermore, these peptides display cytokine-like regulatory properties and can be influenced by hormones such as insulin and angiotensin II as well as growth factors such as TGF- β and TNF- α (Nelson et 10 al., supra; Suzuki et al., 1989, J. Biochem. 106: 736-741; and Lundblad et al., 1996, Crit. Care Med. 24: 820-826;). Endothelin activity is mediated via binding with preferential affinities to two distinct G-coupled receptors, ETA and ETB, in an autocrine/paracrine manner (see, e.g., Hocher et al., 15 1997, Eur. J. Clin. Chem. Clin. Biochem. 35(3): 175-189; Shichiri et al., 1991, J. Cardiovascular Pharmacol. 17: S76-S78).

There are a variety of agonists and antagonists of endothelin receptors (Webb et al., 1997, Medicinal Research 20 Reviews 17 (1): 17-67), which have been used to study the mechanism of action of the endothelins. Because endothelin is known to have powerful vasoconstrictive activity, endothelin antagonists in particular (also termed "endothelin receptor antagonists" in the art) have been studied with 25 regard to their possible role in treating human disease, most notably, cardiovascular diseases such as hypertension, congestive heart failure, atherosclerosis, restenosis, and myocardial infarction (Mateo et al., 1997, Pharmacological Res. 36 (5): 339-351). For example, non-peptide-based 30 endothelin antagonists belonging to the pyrimidinyl sulfonamide family, such as Ro 46-2005 and bosentan, which interact with the endothelin receptor through their aromatic rings, are currently undergoing clinical evaluation for the treatment of hypertension, vascular disease, and congestive 35 heart failure. These antagonists can bind both ETA and ETB with varying affinities and have advantages over peptide-based antagonists because they possess an improved metabolic

stability (Webb et al., supra; and Parris et al., supra). In addition, endothelin antagonists have also been studied with regard to their possible role in the treatment of kidney disease such as impaired renal function in liver cirrhosis and acute renal failure (Gomez-Garre et al., 1996, Kidney Int. 50: 962-972; Hocher et al., supra).

More recently, endothelins and endothelin receptors have been implicated in a number of normal and pathological cell growth processes, e.g., cell cycle progression, cell growth, and cellular development (see, e.g., Parris et al., 1997, Vascular Medicine 2: 31-43; Markewitz et al., supra; Morbidelli et al., 1995, Am. J. Physiol. 269: H686-H695; and Battistini et al. 1993, Peptides 14: 385-399). ET1 and ET3 have been shown to be mitogenic and chemokinetic factors for normal tissues ranging from endothelial and epithelial cells to macrophages (see, e.g., Webb et al., 1997, Medicinal Research Reviews 17 (1): 17-67; and Gomez-Garre et al., supra). In addition, the binding of endothelins to their receptors has been shown to cause DNA synthesis, proliferation and cell mobilization in normal and neoplastic cells (Webb et al., supra; Ziche et al., 1995, Cardiovasc. Pharmacol. 26: S284-S286; and Yamashita et al., 1991, Res. Comm. in Chem. Pathol. and Pharmacol. 74 (3): 363-369).

This potential capability of endothelins to mediate cell growth and cell cycle progression has led to some initial studies of endothelin expression and/or endothelin receptor presence in cancer cells. For example, ET-1 has been shown to be overexpressed in breast cancer and pancreatic cell lines and induces proliferation in breast cancer tissue, ovarian cell lines and prostate tumors (see, e.g., Moriatis et al., 1997, Eur. J. Canc. 33 (4): 661-668; Nelson et al., 1996, Cancer Res. 56: 663-668; Patel et al., 1995, Br. J. Cancer 71: 442-447; Oikawa et al., 1994, Br. J. Cancer 69: 1059-1064; Shichiri et al., supra; and Yamashita et al., supra). In addition, the presence of ETA type receptors, which have a higher affinity for ET1 and ET2, has been demonstrated in ovarian cell lines (Moriatis et al., supra).

and breast cancer tissues (Yamashita et al., supra). One of the few tumors to express ETB receptors that have a similar affinity for all three isoforms of endothelin is melanoma (Yohn et al., 1994, Biochem. Biophys. Res. Comm. 201 (1): 449-457). Interestingly, ETB receptors are highly expressed in primary or recurrent melanomas but less so in metastatic melanomas (Kikuchi et al., 1996, Biochem. Biophys. Res. Comm. 219: 734-739).

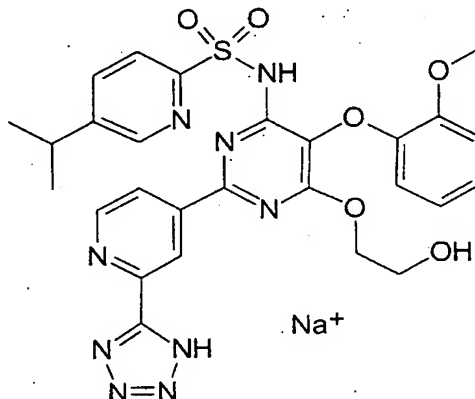
Although these studies suggest that endothelin antagonists could potentially have therapeutic applications in the treatment of cancer, there have been no studies to date demonstrating any such therapeutic application. In fact, the role that endothelin may play in promoting proliferative disease such as various vascular proliferative diseases and benign prostatic hypertrophy (BPH) is unclear (Webb et al., supra and Kenny et al., 1997, J. Med. Chem. 40 (9): 1293-1315). Moreover, while United States Patents 5,550,110 and 5,641,752 disclose the use of specific hexapeptide endothelin antagonists for the treatment of cancer, there is actually no data in those disclosures relating to cancer treatment and no indication as to how to perform such treatment or indeed whether such treatment would be successful (see also, PCT applications WO 97/37987, 97/08169, WO 96/11927, and WO 94/03483, Canadian patent application 2072395, and United States Patent 5,658,943).

3. SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for the treatment of cell proliferative disorders such as cancer. More specifically, the invention relates to compositions comprising at least one endothelin antagonist, preferably in combination with a poly- β -1-4-N-acetylglucosamine (p-GlcNAc) polysaccharide matrix, for use in the treatment of cancer and other proliferative diseases. The present invention is based, in part, on Applicants' discovery that, when an endothelin antagonist is administered in vivo, either alone in high doses or in combination with a

polysaccharide matrix, tumor cell growth and/or the growth or metastasis of neoplastic cells are significantly inhibited.

According to a preferred embodiment of the invention, the endothelin antagonist is a non-peptide-based pyrimidyl sulfonamide compound, such as that depicted in Figure I below.



The compound of Figure I is 5-Isopropyl-pyridine-2-sulfonic acid [6-(2--hydroxy-ethoxy)-5-(2-methoxy-phenoxy),-2-[2-(1H-tetrazol-5-yl)-pyridin-4-yl]-pyrimidin-4-yl]amide sodium salt (1:2), also termed herein "Ro61" and has a molecular weight of approximately 650 kD. It is a non-specific, non-peptide inhibitor of both endothelin receptors, ETA and ETB.

According to a preferred embodiment of the invention, the polysaccharide matrix is a poly- β -1-4-N-acetylglucosamine (p-GlcNAc) polysaccharide matrix, or a derivative thereof, as described in United States Patent 5,635,493, which is incorporated herein by reference in its entirety. The p-GlcNAc or its derivatives may be utilized in various reformulations, including membranes, filaments, non-woven textiles, sponges, gels and three-dimensional matrices. According to a preferred embodiment, the p-GlcNAc is in the form of a gel, is preferably deacetylated and optionally, derivatized to a p-GlcNAc-lactate salt, and is combined with Ro61 for administration in vivo.

The compositions of the invention are useful for drug delivery systems, e.g., slow-release drug delivery. The

compositions of the invention are an improvement over traditional drug formulations in that the compositions of the invention provide, for example, increased effectiveness, reduced toxicity and improved bioavailability.

5 The methods of the invention comprise the administration of therapeutically effective amounts of the compositions of the invention in vivo for the treatment of cell proliferative diseases such as cancer in an animal, including humans. According to one embodiment of the invention, at least one
10 endothelin antagonist, such as Ro61, is dissolved in a deacetylated p-GlcNAc-lactate gel and administered, in a therapeutically effective amount, to a patient in vivo for the treatment of cancer or other proliferative diseases or disorders. Another embodiment of the invention comprises the
15 administration in vivo of an endothelin antagonist, more preferably, a non-peptide-based endothelin antagonist such as a pyrimidyl sulfonamide endothelin antagonist, for the treatment of cancer or other proliferative diseases or disorders. Yet another embodiment of the invention comprises
20 the administration in vivo of a p-GlcNAc matrix alone for the treatment of cancer or other proliferative diseases or disorders. The compositions and methods of the invention are useful for the inhibition of tumor and/or other neoplastic cell growth and/or the inhibition of metastasis of neoplastic
25 cells in vivo.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Chemical structure of 100% p-GlcNAc. "n" refers to an integer ranging from about 4,000 to about
30 150,000, with about 4,000 to about 15,000 being preferred.

FIG. 2. Carbohydrate analysis of p-GlcNAc, Gas Chromatography-Mass Spectroscopy data. Solid squares represent p-GlcNAc purified using the acid treatment/neutralization method described in Section 5.1,
35 infra.

FIG. 3. Scanning electron micrograph depicting a p-GlcNAc membrane prepared by the acid treatment/neutralization

variation of the chemical/biological purification method.
Magnification: 10,000x.

FIG. 4. Diagram depicting some of the possible p-GlcNAc and deacetylated p-GlcNAc derivatives of the invention
5 (Adapted from S. Hirano, In "Chitin and Chitosan", 1989, Skjak-Braek, Anthonsen, and Sanford, eds., Elsevier Science Publishing Co., pp. 37-43).

FIGS. 5A and 5B. Scanning electron micrographs of a deacetylated p-GlcNAc mat. Magnification: FIG. 5A: 1000x;
10 FIG. 5B: 10,000x.

FIGS. 6A and 6B. Scanning electron micrographs of a p-GlcNAc membrane dissolved in dimethylacetamide/lithium chloride and reprecipitated in water into a fibrous material, as described in Example Section 8, infra.

15 FIG. 7. Endothelin receptor antagonist Ro61 inhibition of B16 melanoma cell proliferation in vitro. Ro61 was added at increasing concentrations to a 96 well culture plate to which B16 cells (closed circles) and splenocytes (open circles) from C57BL/6 (H-2b) mice were then added.
20 Proliferation of the Ro61-treated cells is expressed as a percentage of untreated control cells. Mean values of triplicate wells were determined.

FIG. 8. Bar graphs indicating the percent proliferation of B16 melanoma cell relative to untreated controls upon
25 exposure of the cells to various endothelin antagonists. The results indicate an inhibition of proliferation upon exposure of the cells to the endothelin antagonists. FO cells are control B16 cells that lack a full length ETA receptor; the bar graph marked ET1 represents a control, wherein the cells
30 were exposed to a known endothelin agonist.

FIG. 9. Bar graphs indicating the percent proliferation of B16 melanoma cell relative to untreated controls upon
exposure of the cells to various endothelin antagonists. The results indicate an inhibition of proliferation upon exposure
35 to the endothelin antagonists. FO cells are control B16 cells that lack a full length ETA receptor; the bar graphs

marked ET1 and BQ3020 represent controls, wherein the cells were exposed to two known endothelin agonists.

FIG. 10. ETA and ETB agonists reversal of Ro61 inhibition of B16 melanoma cell proliferation in vitro. B16 cells were cultured with either agonist BQ-3020-[Ac-[Ala11,Ala15]-endothelin(6,21) (closed triangle), agonist [Ala^{1,3,11,15}]-endothelin1 (open diamond), both agonists (open box) or neither (closed circle) and Ro61 was then added to each well. Proliferation of the Ro61-treated cells is expressed as a percentage of untreated control cells. Mean values of triplicate wells were determined.

FIGS. 11A and 11B. Effect of Ro61 on B16 cells in culture. Light micrograph of B16 cells at 40x magnification. FIG. A: B16 cells cultured in 96 well plates at 5×10^4 cells/well for 72 hours at 37°C in complete media; FIG. B: B16 cells cultured in complete media containing 5 μ M Ro61.

FIG. 12. Ro61 induces apoptosis. B16 cells were assayed for apoptosis with a Fluorescein In Situ Cell Death Detection Kit after being cultured with either common media (Control) or Ro61 (1 μ M) (■) for 0, 24, 48, and 72 hours at 37°C.

FIGS. 13A and 13B. Ro61 inhibition of B16 intraperitoneal carcinomatosis. FIG. 13A depicts the effect of IP Ro61 on B16 intraperitoneal carcinomatosis. Female C57BL/6 mice were injected IP with 5×10^4 B16 melanoma cells. The next day, animals were injected with 100 μ l of either: daily x 6 HBSS (control), daily x 6 HBSS containing 3 mg/kg of Ro61 (low dose), daily x 6 HBSS containing 30 mg/kg of Ro61 (high dose). FIG. 13B shows the effect of p-GlcNAc delivery of Ro61. Animals were challenged with tumor as in FIG. 13A and treated on the next day with 100 μ l of p-GlcNAc gel injected either IP, SC or IP with 18 mg/kg of Ro61 (IP+Ro61). Animals were sacrificed after 7 days and evaluated for the presence of B16 colonies. Values represent the mean number of visible colonies and standard error for each group (n=11 for all groups except no treatment group, n=13).

FIG. 14. Delayed tumor appearance in Ro61-treated C57BL/6 mice after subcutaneous B16 melanoma tumor challenge.

Female C57BL/6 mice were injected SC with 5×10^4 B16 melanoma cells. The following day, animals were randomly separated into 4 groups: no treatment (\blacktriangle), one IP injection of p-GlcNAc gel alone (O), daily IP injections of 3 mg/kg (for 6 days), Ro61 in HBSS (\square), one IP injection of p-GlcNAc gel containing 18 mg/kg of Ro61 (\blacksquare). Animals were monitored for presence of tumor for 3 weeks ($n = 10$ in all groups).

FIG. 15. Inhibitory effect of various endothelin antagonists on appearance of B16 tumor colonies using carcinomatosis model described infra. F10 represents untreated B16 control cells; BQmix represents a mixture of the ETA antagonist, BQ123 and the ETB antagonist, BQ788; BQmix/gel represents a mixture of BQ123 and BQ788 in combination with the p-GlcNAc gel described infra; Ro61 is the non-specific ETA/ETB endothelin antagonist described infra; Ro61/gel is Ro61 in combination with p-GlcNAc; and GRGDS/gel is a combination of the ETA/ETB peptide endothelin antagonist GRGDS in combination with p-GlcNAc.

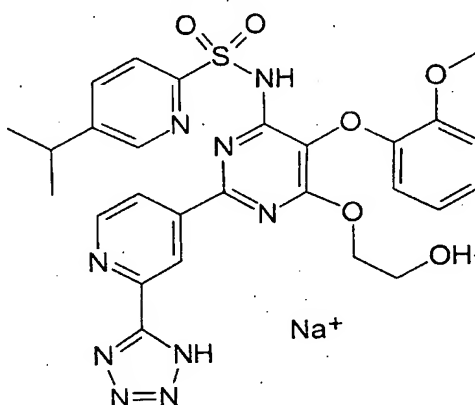
FIG. 16. Long term survival of Ro61-treated C57BL/6 mice after intraperitoneal B16 melanoma challenge. C57BL/6 mice were injected intraperitoneally with B16 cells. Animals were randomly separated into 4 groups for either of the following treatments: (a) no treatment (closed boxes); (b) 100 μ l of p-GlcNAc gel alone (crosses); (c) 100 μ l of daily HBSS containing 3 mg/kg Ro61 (closed triangles); or (d) 100 μ l of p-GlcNAc gel containing 18 mg/kg Ro61 (open boxes). Animals were monitored daily and sacrificed for humane reasons when determined moribund.

FIGS. 17A and 17B. Photomicrographs of the cell morphology of Ro61-treated (FIG. 17A) and untreated (FIG. 17B) B16 cells at 40X power; 10^{-7} M Ro61, 10^5 cells.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions comprising at least one endothelin antagonist, preferably in combination with a poly- β -1-4-N-acetylglucosamine (p-GlcNAc) polysaccharide matrix, and methods for using these

compositions in the treatment of cancer and other proliferative diseases. The endothelin antagonists according to this invention may be specific or non-specific for ETA or ETB receptors or peptide-based or non-peptide-based compounds. According a preferred embodiment of the invention, the endothelin antagonist is a non-peptide-based, non-specific endothelin antagonist. According to another preferred embodiment, the endothelin antagonist is a non-peptide-based pyrimidyl sulfonamide compound, such as the Ro61 compound depicted in Figure I below.



According to the present invention, at least one type of endothelin antagonist, alone or in combination with one or more other antitumor agents, is covalently or non-covalently attached to, or combined with, the p-GlcNAc described in detail in Section 5.1, *infra*. According to one preferred embodiment of the invention, at least one endothelin antagonist, such as Ro61, is dissolved in a deacetylated p-GlcNAc gel to form an endothelin antagonist ("EA")/p-GlcNAc composition of the invention. According to a further preferred embodiment, the deacetylated p-GlcNAc is derivatized with lactic acid to form a p-GlcNAc-lactate salt.

As defined herein, the term "endothelin antagonist" includes endothelin receptor antagonists and "EA/p-GlcNAc compositions" include compositions wherein at least one type of endothelin antagonist is either covalently attached to the p-GlcNAc or is non-covalently bound to, mixed with or encapsulated within the p-GlcNAc. The compositions of the

invention can additionally comprise other antitumor agents, which in combination with the endothelin antagonist, act to inhibit the growth and/or metastasis of tumor or other neoplastic cells. As defined herein, "antitumor agent" includes any compound that inhibits the growth or metastasis of tumor cells, cancer cells, or any other type of neoplastic cell.

This invention is based in part on Applicants' discovery that endothelin antagonists, either alone or in combination with the p-GlcNAc described herein, inhibit the proliferation of neoplastic cells in vitro and decrease metastases and/or increase survival of tumor cell-bearing animals in vivo (See Example Sections 12 through 16, infra). In addition, the p-GlcNAc of this invention alone has an inhibitory effect on metastases and neoplastic cell growth in vivo.

Thus, according to the methods of this invention, pharmaceutical compositions comprising the EA/p-GlcNAc compositions of the invention are administered, in a therapeutically effective amount, to a patient in vivo for the treatment of cancer or other proliferative diseases. Another preferred embodiment of the invention comprises the administration in vivo of an endothelin antagonist, e.g., a pyrimidyl sulfonamide endothelin antagonist for the treatment of proliferative disease. And, yet another embodiment comprises the administration in vivo of the p-GlcNAc described infra for the treatment of proliferative disease.

Solely for ease of description, the detailed description of the invention is divided into the following subsections:

- (1) The p-GlcNAc of the compositions and methods of the invention;
- (2) Endothelin antagonists of the compositions and methods of the invention;
- (3) Preferred formulations of the compositions of the invention; and
- (4) Uses of the compositions and methods of the invention.

5.1 The p-GlcNAc Of The Compositions Of The Invention

The p-GlcNAc polysaccharide matrix to be utilized in the compositions and methods of this invention comprises a
5 polymer of high molecular weight ranging from a weight average of about 800,000 daltons to about 30 million daltons, based upon gel permeation chromatography measurements. Such a molecular weight range represents a p-GlcNAc species having about 4,000 to about 150,000 N-acetylglucosamine
10 monosaccharides attached in a β -1-4 configuration, with about 4,000 to about 15,000 N-acetylglucosamine monosaccharides being preferred (Figure 1).

The variability of the p-GlcNAc is very low, and its purity is very high, both of which are evidenced by chemical
15 and physical criteria. Among these are chemical composition and non-polysaccharide contaminants. First, chemical composition data for the p-GlcNAc produced using two different purification methods is shown in Table I below. As can be seen, the chemical composition of the p-GlcNAc
20 produced by both methods is, within the bounds of experimental error, the same as the formula compositions of p-GlcNAc. Second, as is also shown in Table I, the p-GlcNAc produced is free of detectable protein contaminants, is substantially free of other organic contaminants such as free
25 amino acids, and is substantially free of inorganic contaminants such as ash and metal ions (the p-GlcNAc of the invention may deviate up to about 2% from the theoretical values of carbon, hydrogen, nitrogen and oxygen for pure p-GlcNAc). Therefore, as used herein, the terms "substantially
30 free of organic contaminants" and "substantially free of inorganic contaminants" refer to compositions of p-GlcNAc having the profiles for carbon, hydrogen, nitrogen and oxygen which deviate no more than about 2% from the theoretical values, and preferably, the p-GlcNAc of the invention contain
35 a profile as exemplified in the Experimental Data on p-GlcNAc mats in Table I (allowing for the percent deviation).

Further, the p-GlcNAc exhibits a very low percentage of bound water.

TABLE I

CHEMICAL ANALYSIS DATA (% by weight)

Theoretical Values for Pure p-GlcNAc:

Carbon	-	47.29
Hydrogen	-	6.40
Nitrogen	-	6.89
Oxygen	-	39.41
Protein	-	0.00

Experimental Data on p-GlcNAc Mats:

(Number of experimental batches for each membrane type being greater than 30 for each membrane type)

		<u>MECHANICAL FORCE</u>		<u>CHEMICAL/BIOLOGICAL</u>	
		<u>METHOD</u>		<u>METHOD</u>	
		Normalized ¹	% Dev.	Normalized ¹	% Dev.
20	Carbon	47.21 ± 0.08	-0.17	47.31 ± 0.11	+0.04
	Hydrogen	6.45 ± 0.08	+0.78	6.34 ± 0.08	-0.94
	Nitrogen	6.97 ± 0.18	+0.87	6.94 ± 0.16	+0.73
	Oxygen	39.55 ± 0.36	+0.36	39.41 ± 0.10	0.00
		<u>Average Values</u>		<u>Average Values</u>	
25	Protein	0.00		0.00	
	Ash	1.30		0.98	
	Moisture	2.0		1.2	

¹ Raw analytical data have been normalized to account for ash and moisture content of the samples.

30 The p-GlcNAc of the compositions of the invention exhibits a carbohydrate analysis profile substantially similar to that shown in Figure 2. The primary monosaccharide of the p-GlcNAc is N-acetylglucosamine. Further, the p-GlcNAc does not contain the monosaccharide
 35 glucosamine. Other physical characteristics of the p-GlcNAc are described in detail in United States Patent 5,635,493, which has been incorporated herein by reference.

The p-GlcNAc according to this invention exhibits a high degree of biocompatibility, which may be determined by a variety of techniques, including, but not limited to such procedures as the elution test, intramuscular implantation, or intracutaneous or systemic injection into animal subjects. See, e.g., United States Patent 5,635,493 incorporated herein by reference.

The p-GlcNAc is produced by, and may be purified from, microalgae, preferably diatoms. The diatoms which may be used as starting sources for the production of the p-GlcNAc include, but are not limited to members of the *Coscinodiscus* genus, the *Cyclotella* genus, and the *Thalassiosira* genus, with the *Thalassiosira* genus being preferred.

Among the *Coscinodiscus* genus, the species of diatom that may be used include, but are not limited to the *concinus* and *radiatus* species. The diatoms among the *Cyclotella* genus which may be used include, but are not limited to the *caspia*, *cryptica*, and *meneghiniana* species. The *Thalassiosira* diatoms that may be utilized to produce the starting material for the p-GlcNAc of this invention include, but are not limited to the *nitzschoides*, *aestivalis*, *antarctica*, *decipiens*, *eccentrica*, *floridana*, *fluviatilis*, *gravida*, *guillardii*, *hyalina*, *minima*, *nordenskioldii*, *oceanica*, *polychorda*, *pseudonana*, *rotula*, *tubifera*, *tumida*, and *weissflogii* species, with the *fluviatilis* and *weissflogii* species being preferred. Diatoms such as those described above may be obtained, for example, from the culture collection of the Bigelow Laboratory for Ocean Sciences, Center for Collection of Marine Phytoplankton (McKown Point, West Boothbay Harbor, Maine, 04575). Any of these diatoms may be grown utilizing the methods and nutrient medium described in United States Patent 5,635,493 incorporated herein by reference.

p-GlcNAc fibers may be obtained from diatom cultures such as those described above via a number of different methods. According to the Mechanical Force method, p-GlcNAc fibers may be separated from diatom cell bodies by subjecting

the contents of the culture to an appropriate mechanical force. Such a mechanical force may include, but is not limited to, a shear force generated by, for example, a colloid mill, an ultrasound device, or a bubble generator, or
5 a cutting force generated by, for example, a Waring blender.

The resulting suspension of diatom cell bodies and p-GlcNAc fibers are then segregated. For example, the suspension may be subjected to a series of centrifugation steps which segregate the p-GlcNAc fibers from the cell
10 bodies, yielding a clear supernatant exhibiting little, if any, visible flocculent material. A fixed angle rotor, and a temperature of about 10° C are preferred for the centrifugation steps. The speed, duration, and total number of centrifugation steps required may vary depending on, for
15 example, the specific centrifugation rotor being used, but the determination of the values for such parameters will be apparent to one of ordinary skill in the art.

The p-GlcNAc fibers in the supernatant may then be concentrated using techniques well known to those of skill in
20 the art. Such techniques may include, but are not limited to suction and filtration devices. Finally, the concentrated p-GlcNAc fibers are washed with, for example, distilled-deionized water, HCl and ethanol, or other appropriate solvents, preferably solvents, such as alcohols, in which
25 both organic and inorganic materials dissolve. An example demonstrating the use of this method for the purification of p-GlcNAc is set forth in Example Section 6, infra.

According to the Chemical/Biological Method, p-GlcNAc fibers are separated from diatom cell bodies by subjecting
30 them to chemical and/or biological agents. For example, diatom cultures may be treated with a chemical capable of weakening diatom cell walls, which leads to a release of the p-GlcNAc fibers without altering their structure. Such a chemical may include, but is not limited to, hydrofluoric
35 acid (HF). Alternatively, a mature diatom culture may be treated with a biological agent capable of altering a biological process and may be used to inhibit p-GlcNAc fiber

synthesis, thus releasing the fibers already present. For example, such an agent may include, but is not limited to, polyoxin-D, an inhibitor of the enzyme N-acetylglucosaminyl-P-transferase.

5 The cell bodies and p-GlcNAc-containing fibers of diatom cultures treated with a member of the above described chemical or biological agents are then segregated. For example, the contents of treated diatom cultures may be allowed to settle such that the contents of the cultures are
10 allowed to form two distinct layers. The upper layer will contain primarily the p-GlcNAc fibers, while the bottom layer will contain the cell bodies. The upper p-GlcNAc fiber-containing layer may be siphoned off, leaving behind the settled cellular material of the bottom layer. The siphoned
15 off p-GlcNAc fiber-containing layer may then be further purified to remove protein and other unwanted matter by treatment with a detergent that will not damage the p-GlcNAc fibers. Such a detergent may include, but is not limited to, sodium dodecyl sulfate (SDS).

20 When acid treatment, such as HF treatment, is used to separate p-GlcNAc fibers from diatom cell bodies, a step may be included for the dispersal of the fibers. Such a step may include, but is not limited to, the use of mechanical force for fiber dispersal, such as a step in which the fibers are
25 subjected to the movements of an orbital shaker.

Alternatively, the acid-treated suspension may, in an optional step, be neutralized prior to further purification by detergent treatment. Such neutralization will, in general, change the pH of the suspension from approximately
30 1.8 to approximately 7.0, and may be accomplished by, for example, the addition of an appropriate volume of 1M Tris (pH 8.0) or the addition of an appropriate volume of sodium hydroxide (NaOH). Neutralization, in general, yields pure p-GlcNAc fibers of a substantially greater length than the
35 other purification methods discussed herein.

The purified p-GlcNAc fibers may then be concentrated using techniques well known to those of skill in the art,

such as by utilizing a suction and filtration device.
Finally, the p-GlcNAc fibers are washed, in a series of steps
with distilled-deionized water, HCl and ethanol, or other
appropriate solvents, preferably solvents, such as alcohols,
5 in which both organic and inorganic materials dissolve. An
example demonstrating the successful utilization of such a
purification method is set forth in Example Section 7, infra.

While each of these methods for the purification of p-
GlcNAc from microalgae, preferably diatom, starting sources
10 produces very pure, unadulterated, crystalline p-GlcNAc, each
of the methods yields p-GlcNAc having specific
characteristics and advantageous features. For example, the
p-GlcNAc purified via the Mechanical Force method produces a
p-GlcNAc membrane that provides a superior substrate for the
15 attachment of cells to the p-GlcNAc. The Chemical/Biological
method produces a much higher average yield than the average
p-GlcNAc yield produced by the Mechanical Force method.
Additionally, the acid treatment/neutralization variation of
the Chemical/Biological method produces extremely long p-
20 GlcNAc fibers, with some fibers being in excess of 100 μ m,
and containing molecules of the p-GlcNAc polymer of very high
molecular weight, as high as 20-30 million daltons.

The electron micrographic structure of the p-GlcNAc to
be utilized in the compositions and methods of this
25 invention, produced using the acid treatment/neutralization
variation of the chemical/biological purification method is
depicted in Figure 3. Purification of the p-GlcNAc fibers
often results in the formation of fibrous membranes as
depicted in Figure 3.

30

5.1.1 Derivatization Of p-GlcNAc

The fully acetylated p-GlcNAc of the invention may be
derivatized, by utilizing a variety of controlled conditions
and procedures, into a large range of different compounds.
35 See Figure 4 for a diagram depicting some of these compounds.
Such derivatized compounds may include, but are not limited
to, partially or completely deacetylated p-GlcNAc, which has

been modified via chemical and/or enzymatic means as described in further detail below. According to a preferred embodiment of the invention, the p-GlcNAc is a 100% deacetylated p-GlcNAc.

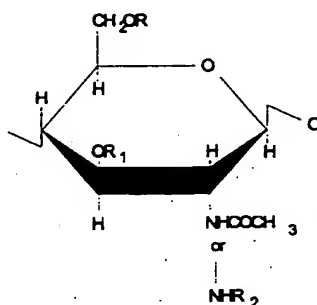
5 Additionally, p-GlcNAc, or its deacetylated derivative, may be derivatized by being sulfated, phosphorylated, and/or nitrated. Further, as detailed below, O-sulfonyl, N-acyl, O-alkyl, N-alkyl, deoxyhalogen, and N-alkylidene and N-arylidene and other derivatives may be prepared from the p-
10 GlcNAc or deacetylated p-GlcNAc of the invention. The deacetylated p-GlcNAc of the invention may also be used to prepare a variety of organic salts and/or metal chelates.

 According to a preferred embodiment of the invention, one or more of the monosaccharide units of the p-GlcNAc may
15 be deacetylated to form a deacylated poly- β -1-4-N-glucosamine species. A poly- β -1-4-N-glucosamine species in which each of the monosaccharide units of the poly- β -1-4-N-acetylglucosamine species has been deacetylated, i.e., a 100% deacetylated derivative, will have a molecular weight of
20 about 640,000 daltons to about 24 million daltons, with about 640,000 daltons to about 2.4 million daltons being preferred. A species with such a molecular weight range represents a species having about 4000 to about 150,000 glucosamine monosaccharides covalently attached in a β -1-4 configuration.

25 The p-GlcNAc may be deacetylated by treatment with a base to yield glucosamines with free amino groups. This hydrolysis process may be carried out with solutions of concentrated sodium hydroxide or potassium hydroxide at elevated temperatures. See, e.g., Example Section 8, *infra*.
30 Alternatively, an enzymatic procedure utilizing a chitin deacetylase enzyme may be used for p-GlcNAc deacylation. Such a deacetylase enzymatic procedure is well known to those of skill in the art and may be performed as in U.S. Patent No. 5,219,749, which is incorporated herein, by reference, in
35 its entirety.

 Further, one or more of the monosaccharide units of the p-GlcNAc of the invention may be derivatized to contain at

least one sulfate group, or, alternatively, may be phosphorylated or nitrated, as depicted below:



where, R and/or R_1 , in place of a hydrogen, and/or R_2 , in place of $-\text{COCH}_3$, may be a sulfate ($-\text{SHO}_3$), a phosphate ($-\text{P}(\text{OH})_2$), or a nitrate ($-\text{NO}_2$) group.

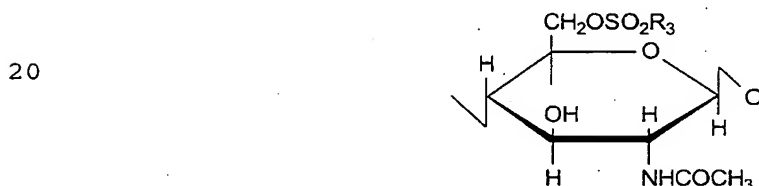
15. Described below are methods by which such p-GlcNAc derivatives may be prepared. Before performing these methods, it may be advantageous to first lyophilize, freeze in liquid nitrogen, and pulverize the p-GlcNAc starting material.
- 20 Sulphated p-GlcNAc derivatives may be generated, by, for example, a two step process. In the first step, O-carboxymethyl p-GlcNAc may be prepared from the p-GlcNAc and/or p-GlcNAc derivatives of the invention by, for example, utilizing techniques such as those described by Tokura et al. (Tokura, S. et al., 1983, Polym. J. 15:485). Second, the
- 25 sulfation step may be carried out with, for example, N, N-dimethyl-formamide-sulfur trioxide, according to techniques well known to those of skill in the art, such as are described by Schweiger (Schweiger, R.G., 1972, Carbohydrate Res. 21:219). The resulting product may be isolated as a
- 30 sodium salt.

Phosphorylated p-GlcNAc derivatives may be prepared, for example, by utilizing techniques well known to those of skill in the art, such as those described by Nishi et al. (Nishi, N. et al., 1986, in "Chitin in Nature and Technology, Muzzarelli et al., eds. Plenum Press, New York, pp. 297-299). Briefly, a p-GlcNAc/methanesulfonic acid mixture may be

treated with phosphorus pentoxide (in an approximately 0.5 to 4.0 molar equivalent) with stirring, at a temperature of about 0° C to about 5° C. Treatment may be for about 2 hours. The resulting product may then be precipitated and washed
5 using standard techniques well known to those of skill in the art. For example, the sample may be precipitated with a solvent such as ether, centrifuged, washed with a solvent such as ether, acetone, or methanol, and dried.

Nitrated p-GlcNAc derivatives may be prepared by
10 utilizing techniques well known to those of skill in the art, such as those described by Schorigin and Halt (Schorigin, R. and Halt, E., 1934, Chem. Ber. 67:1712). Briefly, p-GlcNAc and/or a p-GlcNAc derivative may be treated with concentrated nitric acid to form a stable nitrated product.

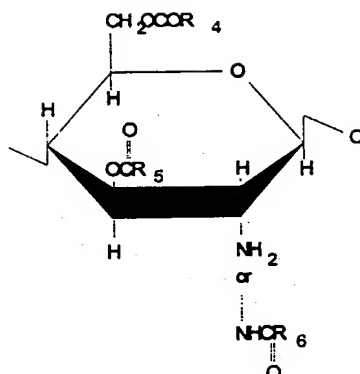
15 One or more of the monosaccharide units of the p-GlcNAc of the invention may contain a sulfonyl group, as depicted below:



25 where R_3 may be an alkyl, an aryl, an alkenyl, or an alkynyl moiety. Such a derivative may be generated by well known methods such as the method described in Kurita et al.

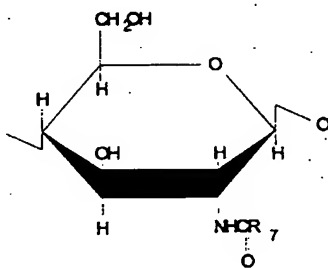
(Kurita, K. et al., 1990, Polym. Prep [Am. Chem. Soc., Div. Polym. Chem.] 31:624-625). Briefly, an aqueous alkali p-
30 GlcNAc solution may be reacted with a chloroform solution of tosyl chloride, and the reaction may then be allowed to proceed smoothly at low temperatures.

One or more of the monosaccharides of the p-GlcNAc of the invention or its deacetylated derivative may contain one
35 or more O-acyl groups, as depicted below:



where R_4 and/or R_5 , in place of hydrogen, may be an alkyl, an alkenyl, or an alkynyl moiety, and R_6 may be an alkyl, an alkenyl, or an alkynyl moiety. An example of such a derivative may be generated by well known methods such as those described by Komai (Komai, T. et al., 1986, in "Chitin in Nature and Technology", Muzzarelli et al., eds., Plenum Press, New York, pp. 497-506). Briefly, p-GlcNAc may be reacted with any of a number of suitable acyl chlorides in methanesulfonic acid to yield p-GlcNAc derivatives which include, but are not limited to, caproyl, capryl, lanoyl, or benzoyl derivatives.

One or more of the monosaccharides of the deacetylated p-GlcNAc of the invention may contain an N-acyl group, as depicted below:

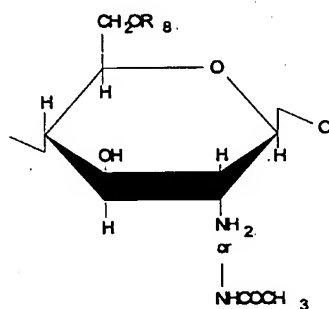


where R_7 may be an alkyl, an alkenyl, or an alkynyl moiety.

Such a derivatization may be obtained by utilizing techniques well known to those of skill in the art, such as the technique described in Hirano et al. (Hirano, S. et al.,

1976, Carbohydrate Research 47:315-320). Deacetylated p-GlcNAc is soluble in a number of aqueous solutions of organic acids. The addition of selected carboxylic anhydrides to such p-GlcNAc-containing solutions, in aqueous methanolic
 5 acetic acid, results in the formation of N-acyl p-GlcNAc derivatives. N-acyl p-GlcNAc is a preferred derivative for the production of controlled release drug delivery systems.

One or more of the monosaccharides of the p-GlcNAc of the invention or of its deacetylated derivative, may contain
 10 an O-alkyl group, as depicted below:

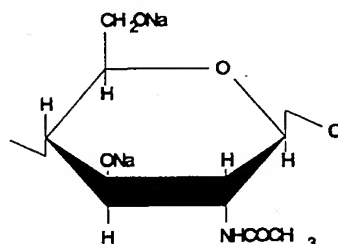


15

20

where R₈ may be an alkyl, and alkenyl, or a alkynyl moiety. Such a derivatization may be obtained by using techniques well known to those of skill in the art. For example, the procedure described by Maresh et al. (Maresh, G. et al., in
 25 "Chitin and Chitosan," Skjak-Braek, G. et al., eds., 1989, Elsevier Publishing Co., pp. 389-395). Briefly, deacetylated p-GlcNAc may be dispersed in dimethoxyethane (DME) and reacted with an excess of propylene oxide. The period of the reaction may be 24 hours, and the reaction takes place in an
 30 autoclave at 40 to 90° C. The mixture may then be diluted with water and filtered. The DME may be removed by distillation. Finally, the end-product may be isolated via lyophilization. The O-alkyl p-GlcNAc and its deacetylated derivative is also a preferred derivative for the production
 35 of controlled release drug delivery systems.

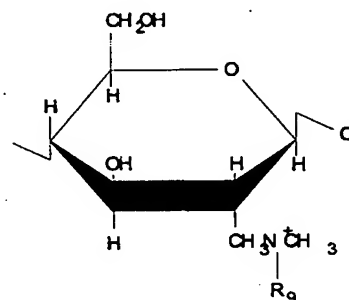
One or more of the monosaccharide units of the p-GlcNAC of the invention may be an alkali derivative, as depicted below:



Such a derivative may be obtained by using techniques well known to those of skill in the art. For example, a method such as that described by Noguchi et al. (Noguchi, J. et al., 1969, Kogyo Kagaku Zasshi 72:796-799) may be utilized.

15 Briefly, p-GlcNAC may be steeped, under vacuo, in NaOH (43%, preferably) for a period of approximately two hours at about 0°C. Excess NaOH may then be removed by, for example, centrifugation in a basket centrifuge and by mechanical pressing.

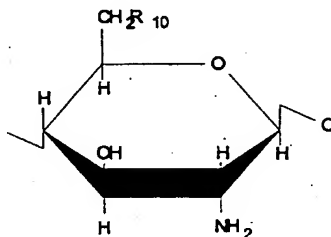
20 One or more of the monosaccharide units of the deacetylated derivative of the p-GlcNAC of the invention may contain an N-alkyl group, as depicted below:



where R₉ may be an alkyl, an alkenyl, or an alkynyl moiety. Such a derivatization may be obtained by utilizing, for example, a procedure such as that of Maresh et al. (Maresh, G. et al., in "Chitin and Chitosan," Skjak-Brack, G. et al., eds. 1989, Elsevier Publishing Co., pp. 389-395), as

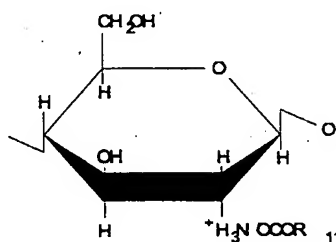
described, above, for the production of N-alkyl p-GlcNAC derivatives.

One or more of the monosaccharide units of the deacetylated derivative of the p-GlcNAC of the invention may contain at least one deoxyhalogen derivative, as depicted below:



where R_{10} may be F, Cl, Br, or I, with I being preferred. Such a derivative may be obtained by using techniques well known to those of skill in the art. For example, a procedure such as that described by Kurita et al. (Kurita, K. et al., 1990, Polym. Prep. [Am. Chem. Soc. Div. Polym. Chem.] 31:624-625) may be utilized. Briefly, a tosylated p-GlcNAC is made to react with a sodium halide in dimethylsulfoxide, yielding a deoxyhalogen derivative. p-GlcNAC tosylation may be performed by reacting an aqueous alkali p-GlcNAC solution with a chloroform solution of tosyl chloride. Such a reaction may proceed smoothly at low temperatures.

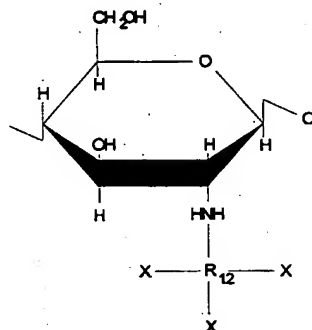
One or more of the monosaccharide units of the deacetylated derivative of the p-GlcNAC of the invention may form a salt, as depicted below:



where R_{11} may be an alkyl, an alkenyl, or an alkynyl moiety. Such a derivatization may be obtained by using techniques

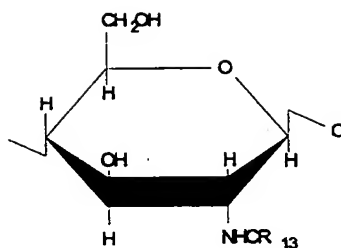
well known to those of skill in the art. For example, a procedure such as that described by Austin and Sennett (Austin, P.R. and Sennett, S., in "Chitin in Nature and Technology," 1986, Muzzarelli, R.A.A. et al., eds. Plenum Press, pp. 279-286) may be utilized. Briefly, deacetylated p-GlcNAc may be suspended in an organic medium such as, for example, ethyl acetate or isopropanol, to which may be added an appropriate organic acid such as, for example, formic, acetic, glycolic, or lactic acid. The mixture may be allowed to stand for a period of time (1 to 3 hours, for example). The temperature of reaction and drying may vary from about 12° to about 35°C, with 20° to 25°C being preferred. The salts may then be separated by filtration, washed with fresh medium, and the residual medium evaporated.

One or more of the monosaccharide units of the deacetylated derivative of the p-GlcNAc of the invention may form a metal chelate, as depicted below:



where R_{12} may be a metal ion, particularly one of the transition metals, and X is the dative bond established by the nitrogen electrons present in the amino and substituted amino groups present in the deacetylated p-GlcNAc.

One or more of the monosaccharide units of the deacetylated derivative of the p-GlcNAc of the invention may contain an N-alkylidene or an N-arylidene group, as depicted below:



5

where R_{13} may be an alkyl, an alkenyl, an alkynyl, or an aryl moiety. Such a derivatization may be obtained by using techniques well known to those of skill in the art. For example, a procedure such as that described by Hirano et al. (Hirano, S. et al., 1981, J. Biomed. Mat. Res. 15:903-911) may be utilized. Briefly, an N-substitution reaction of deacetylated p-GlcNAc may be performed with carboxylic anhydrides and/or arylaldehydes to yield acyl- and/or arylidene derivatives.

Further, the p-GlcNAc, or its deacetylated derivative, may be subjected to controlled hydrolysis conditions, which yield groups of molecules having uniform, discrete molecular weight and other physical characteristics. Such hydrolysis conditions may include, for example, treatment with the enzyme, lysozyme. p-GlcNAc may be exposed to lysozyme for varying periods of time, in order to control the extent of hydrolysis. In addition, the rate of hydrolysis may be controlled as a function of the extent to which the p-GlcNAc that is being lysozyme-treated has been deacetylated. Deacetylation conditions may be as described *supra*. The more fully a p-GlcNAc molecule has been deacetylated, between about 20 and about 90 percent deacetylated, the more fully the molecule will be hydrolyzed in a given time. Changes in physical characteristics, in addition to the lowering of molecular weight, may be elicited by hydrolysis and/or deacetylation treatments. Extensive hydrolysis causes liquefaction of the p-GlcNAc.

Further, heat denaturation may function to modify the crystalline structure of the p-GlcNAc. Such a modification of the p-GlcNAc product crystalline structure may

advantageously affect, for example, the reactivity of the p-GlcNAc.

In addition, hybrids comprising p-GlcNAc and/or p-GlcNAc derivatives may be formed. Such hybrids may contain any of a number of natural and/or synthetic materials, in addition to p-GlcNAc and/or p-GlcNAc derivatives. For example, hybrids may be formed of p-GlcNAc and/or p-GlcNAc derivatives plus one or more extracellular matrix (ECM) components. Such ECM components may include, but are not limited to, collagen, fibronectin, glycosaminoglycans, and/or peptidoglycans. Hybrids may also be formed of p-GlcNAc and/or p-GlcNAc derivatives plus one or more synthetic materials such as, for example, polyethylene. Such a p-GlcNAc/polyethylene or p-GlcNAc derivative/polyethylene hybrid may be made by thermally linking the hybrid components via, for example, autoclaving.

Preferred p-GlcNAc derivatives for use in the claimed invention are deacetylated p-GlcNAc salt derivatives such as a p-GlcNAc-lactate derivative, especially a p-GlcNAc-lactate gel derivative. As used herein, the term "p-GlcNAc-lactate" means that the lactic acid moiety is functionally attached to a partially or fully deacetylated p-GlcNAc. Such p-GlcNAc-lactate derivatives may be obtained as described above (e.g., by derivatization with lactic acid) and formulated as a gel using propylene glycol and water, as described in Example Section 10, *infra*. p-GlcNAc-lactate derivatives may be produced having high and low viscosities, which allows for the ability to tailor the p-GlcNAc to the specific indication of interest. For example, it may be useful to use a p-GlcNAc having a lower viscosity for delivery through a syringe or via a spray.

As described in greater detail in Section 5.3, *infra*, the p-GlcNAc and/or its derivatives as described above, can be further derivatized by the covalent or non-covalent attachment to, or combination with, molecules or drugs of interest such as endothelin antagonists.

5.1.2 Reformulations Of p-GlcNAc

The p-GlcNAc, its deacetylated derivatives and/or their derivatizations, such as those described above, to be used in the compositions of the invention, may be dissolved and subsequently reformulated into a variety of shapes and configurations.

Solution of the p-GlcNAc can be achieved by treatment with dimethyl acetamide (DMA)/lithium chloride. p-GlcNAc may be readily dissolved by stirring in a DMA solution containing 5% LiCl (by weight of the DMA). Water-soluble p-GlcNAc derivatives, such as p-GlcNAc salts, e.g., lactate or carboxymethyl derivatives, may be dissolved in water. p-GlcNAc which has been at least about 75% deacetylated may be put into solution in, for example, a mild acidic solution, such as 1% acetic acid. p-GlcNAc derivatives that are water-insoluble may be put into solution in organic solvents.

Derivatization of p-GlcNAc in DMA:LiCl with phenyl isocyanates may be used to produce carbanilates. Further, derivatization of p-GlcNAc in DMA:LiCl with toluene-p-sulphonylchloride may be used to produce toluene-p-sulfonate.

The p-GlcNAc, its deacetylated derivatives, and/or their derivatizations in solution may then be precipitated and reformulated into shapes which include, but are not limited to, mats, strings, microspheres, microbeads, membranes, fibers, powders, sponges and gels. Further, ultrathin (i.e., less than about 1 micron thick) uniform membranes may be formulated. Additionally, pharmaceutical formulations such as pills, tablets and capsules can be prepared.

Such reformulations may be achieved, by, for example, taking advantage of the fact that pure p-GlcNAc is insoluble in solutions such as water and alcohol, preferably ethanol. Introduction, by conventional means, such as by injection, for example, of the p-GlcNAc-containing DMA/LiCl mixture into such a water or alcohol, preferably ethanol, solution will bring about the reprecipitation, and therefore reformulation, of the dissolved p-GlcNAc. The reformulation of a p-GlcNAc membrane into a fibrous material is demonstrated in Example

Section 9, infra. In the case of water-soluble p-GlcNAc derivatives, reformulations may be achieved by reprecipitating in such organic solvents as, for example, ethyl acetate or isopropanol. Reformulations of p-GlcNAc which has been at least about 75% deacetylated may be achieved by reprecipitating in an alkaline solution. Water-insoluble p-GlcNAc derivatives may be reformulated by reprecipitation in aqueous solutions, such as, for example, water.

p-GlcNAc membranes and three-dimensional p-GlcNAc matrices may be produced via methods which provide for the formation of controlled average pore sizes within either the membranes or the matrices. Pore size can be controlled in membranes and matrices by varying the amount of p-GlcNAc material used, and by the addition of certain solvents such as methanol or ethanol, with ethanol being preferred, in specific amounts, ranging from about 5% to about 40%, prior to the formation of membranes and/or matrices. In general, the greater the percentage of solvent, the smaller the average pore size formed will be.

According to a preferred reformulation of the invention, a p-GlcNAc lactate derivative is formulated into a gel as described in detail in Example Section 10, infra.

5.2 The Endothelin Antagonists Of The Compositions Of The Invention

The endothelin antagonists to be utilized in the compositions and methods of the invention include but are not limited to peptide-based endothelin antagonists, non-peptide-based endothelin antagonists, ETA-specific, ETB-specific, or non-specific endothelin antagonists. Examples of peptide-based endothelin receptor antagonists useful in the compositions and methods of the invention include BQ-123 (Cyclo(-D-Trp-D-Asp-L-Pro-D-Val-L-Leu-), BQ-153, BQ-238, BQ-485, BQ-610, BQ-788, BQ-928, TAK-044, FR139317 (Perhydroazepin-1-ylcarbonyl-L-leucyl-(1-methyl)-D-tryptophyl-[3-(2-pyridyl)]-D-alanine), RES-701-1 (Novabiochem), PD 142893 (Acetyl-(3,3-diphenyl-D-alanine)-L-

Leu-L-Asp-L-Ile-L-Ile-L-Trp), PD 145065, CP 170687, Ac-DBhg16-Leu-Asp-Ile, IRL-1038 ([Cys11-Cys15]-Endothelin-1 (11-21)), the GRGDS pentapeptide, and ET-1[Dpr1-Asp 15]. Many of these peptides can be obtained commercially, e.g., from the American Peptide Company, Sunnyvale CA or Calbiochem-Novabiochem Company, San Diego CA.

Examples of non-peptide-based endothelin receptor antagonists for use in the compositions and methods of the invention include Ro 61-0612, Ro 61-1790, Ro 42-2005, Ro 46-2005, Ro 46-8443, Ro 47-0203 (also known in the art as bosentan), PD 155080, PD 156707, SB 209670, SB 217242, L-744,453, L-749,329, L-754,142, CGS 27830, BMS 182874, LU 135252, S-1039, mA386, A-127722,, TBC11251, Nz-arg-3-(isoxazdylsulfameyl)-2-thiophenecarboxamide, and EQ 123. See, e.g., Webb et al., supra and Ohlstein et al., supra, for structures of many of these known endothelin antagonists.

Non-peptide-based endothelin antagonists may be preferred according to this invention because they display more favorable pharmacokinetic properties than peptide-based antagonists, e.g., enhanced metabolic stability and better bioavailability and oral activity. According to a preferred embodiment of the invention, the endothelin antagonist utilized is Ro61 as depicted in Figure I, supra.

25

5.3 Preferred Formulations Of The Compositions Of The Invention

According to a preferred embodiment of the invention, an endothelin antagonist as described supra ("EA") is covalently or non-covalently functionally attached to, or combined with, the p-GlcNAc, or one or more derivatives or reformulations thereof, as described supra. According to one embodiment, at least one type of endothelin antagonist is covalently, non-covalently or otherwise combined or mixed with a deacetylated p-GlcNAc. Other antitumor agents which may be used in conjunction with the EA/p-GlcNAc compositions of the invention are discussed infra.

35

The endothelin antagonist or other antitumor agent may be covalently attached to the exposed primary amines of deacetylated p-GlcNAc by, for example, chemical attachment utilizing bi-functional cross-linking reagents that act as
5 specific-length chemical spacers. Such techniques are well known to those of skill in the art, and may resemble, for example, the methods of Davis and Preston (Davis, M. and Preston, J.F. 1981, Anal. Biochem. 116:404-407) and Staros et al. (Staros, J. V. et al., 1986, Anal. Biochem. 156:220-222).
10 For example, in the case of peptide-based compounds, carboxylic residues on the peptide to be attached to the deacetylated or partially deacetylated p-GlcNAc may be activated and then crosslinked to the p-GlcNAc. Activation may be accomplished, for example, by the addition of a
15 solution such as carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) to a peptide solution in a phosphate buffer. Preferably, this solution would additionally contain a reagent such as sulpho-NHS (N-hydroxysulphosuccinimide) to enhance coupling. The activated
20 peptide may be crosslinked to the deacetylated p-GlcNAc by mixing in a high pH buffer, such as carbonate buffer (pH 9.0-9.2).

The biological activity of the attached molecule can be maintained by varying the length of the linker molecule
25 (e.g., the bi-functional crosslinking compound) utilized to attach the molecule to the p-GlcNAc. An appropriate linker length for a given molecule to be attached which will not alter the biological activity of the attached molecule can routinely be ascertained. For example, the biological
30 activity (e.g., a therapeutically effective level of biological activity) of a molecule which has been attached via a linker of a given length can be tested by utilizing well-known assays specific for the given molecule being attached. Additionally, in order to maintain the biological
35 activity of the molecule being attached, it may be necessary to utilize a linker which can be cleaved by an appropriate naturally occurring enzyme to release the attached molecule.

Assays commonly employed by those of skill in the art may be used to test for the retention of the biological activity of the particular molecule being attached to ensure that an acceptable level of activity (e.g., a therapeutically effective level activity) is retained.

Alternatively, peptide-based or non-peptide-based endothelin antagonists, alone or in combination with other antitumor agents, may be mixed with or non-covalently attached to p-GlcNAc and/or its derivatives to form the compositions of the invention, using techniques well known to those of skill in the art. For example, a molecule or molecules of choice, e.g., an endothelin antagonist, may be mixed with suspensions of p-GlcNAc, with a deacetylated or partially deacetylated p-GlcNAc solution, with a deacetylated or partially deacetylated p-GlcNAc salt solution, e.g. with a p-GlcNAc-lactate solution (partially or fully deacetylated), or with any p-GlcNAc derivative solution. The mixtures may optionally be lyophilized. Molecules become non-covalently bound to the p-GlcNAc matrices following lyophilization, presumably via hydrophobic, electrostatic and other non-covalent interactions. Such p-GlcNAc formulations are very easy to produce. Further, such formulations can effectively be achieved with a wide variety of molecules having a broad spectrum of physical characteristics and water solubility properties, ranging from the most hydrophobic to the most hydrophilic. Upon attachment of the molecule or molecules, assays commonly employed by those of skill in the art to test the activity of the particular non-covalently attached molecule or molecules can be used to ensure that an acceptable level of activity (e.g., a therapeutically effective activity) is achieved with the attached molecule.

In addition, endothelin antagonists, alone or in combination with other antitumor agents, can be encapsulated in the p-GlcNAc using methods known in the art. For example, one method for achieving encapsulation can involve the procedure outlined by Hwang et al. (Hwang, C. et al. in Muzzarelli, R. et al., eds., 1985, "Chitin in Nature and

Technology", Plenum Press, pp. 389-396) which is incorporated by reference in its entirety. Encapsulation can also be achieved, for example, by following a modification of the acid treatment/neutralization variation of the chemical/biological purification method presented above. Rather than raising the pH of the p-GlcNAc solution to approximately neutral pH range (i.e., approximately 7.4), one may create a basic pH environment, by raising the pH to approximately 9.0 after the purification of the p-GlcNAc is completed. At a more basic pH, the structure of the p-GlcNAc, or a derivative thereof, assumes a more three-dimensional or "open" configuration. As the pH is lowered, the molecule's configuration reverts to a more compact, "closed" configuration. Thus, a compound or drug of interest, such as an endothelin antagonist, may be added to a p-GlcNAc solution at a high pH, then the pH of the p-GlcNAc/drug suspension may be lowered, thereby "trapping" or encapsulating the drug of interest within a p-GlcNAc matrix. Upon encapsulation of the molecule, assays commonly employed by those of skill in the art may be utilized to test the activity of the particular molecule or molecules encapsulated, thereby ensuring that an acceptable level of biological activity (e.g., a therapeutically effective activity) is retained by the encapsulated molecule.

25 An example of the preparation of an EA/p-GlcNAc composition of the invention is set forth in Example Section 10, infra, wherein an endothelin antagonist is mixed with a p-GlcNAc lactate gel. Alternatively, an EA composition (without an accompanying p-GlcNAc) can be prepared, e.g., by dissolving the endothelin antagonist in PBS, HBSS or as described by manufacturer's instructions, and adjusting the solution to the desired concentration.

35 The compositions of the invention, including EA/p-GlcNAc compositions, may be formulated for administration as pharmaceutical compositions, e.g., by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration. According to a

preferred embodiment, the EA/p-GlcNAc composition of the invention is administered by injection in the form of a gel as described in Example Section 10, infra. In the embodiment of the invention wherein the pharmaceutical composition
5 comprises the administration of an endothelin antagonist, e.g., a non-peptidyl endothelin antagonist such as a pyrimidyl sulfonamide, for the treatment of proliferative disease, e.g., cancer, the composition may comprise a therapeutically effective amount of the endothelin antagonist
10 in combination with a pharmaceutically acceptable carrier.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients or carriers such as binding agents
15 (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch
20 glycolate); or wetting agents (e.g., sodium lauryl sulphate). The p-GlcNAc may be used in place of, or in addition to, the excipients, carriers and fillers. Tablets may be coated using p-GlcNAc using methods well known in the art.

Liquid preparations for oral administration may take the
25 form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicles before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending
30 agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or
35 sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

5.4 Uses Of The Compositions And Methods Of The Invention

Biomedical uses of the compositions of the invention include their use as drug delivery systems for endothelin antagonists as well as other therapeutic agents such as other antitumor agents. The p-GlcNAc-containing formulations of the invention provide additional benefits compared to known drug formulations, including, for example, increased effectiveness, reduced toxicity and improved bioavailability. In fact, there are numerous advantages in using the p-GlcNAc-based drug delivery systems of the invention. For example, traditional drug administration by injection is commonly used with proteins and many other drugs. However, repeated doses lead to oscillating blood drug concentrations and affect patient comfort and compliance. Oral administration can be advantageous since it allows for a more varied load of the drug to be released and is less discomfoting to the patient. However, proteins and other compounds are denatured and degraded in the stomach.

An improved oral administration, however, is achieved by the p-GlcNAc-containing compositions of the invention by providing a protective environment for the drug once administered. For example, the p-GlcNAc protects a peptide-based endothelin antagonist from the acidic and enzymatic environment of the stomach. The p-GlcNAc system releases the compound via diffusion and/or encapsulation degradation once it reaches the intestinal region, where it is effectively absorbed into the blood stream. These p-GlcNAc systems of the invention can be used, for example, to deliver proteins as well as many other compounds. Liposomes coated with p-GlcNAc derivatives or p-GlcNAc derivatives-alginate encapsulations are preferred for such oral delivery methods.

In addition, upon introduction of the compositions of the invention into a patient, the p-GlcNAc biodegrades over time, such that the attached or enclosed compounds are gradually released into the bloodstream of the patient, thus providing a method for controlled, slow-release drug delivery.

Deacetylated or partially deacetylated p-GlcNAc species may be produced having a predictable rate of biodegradability. For example, the percentage of deacetylation affects the rate at which the p-GlcNAc species
5 degrades. Generally, the higher the percentage of deacetylation, the faster the rate of biodegradability and resorption will be. Thus, the degree of p-GlcNAc biodegradability and the in vivo rate of resorption may be controlled during the p-GlcNAc's production.

10 p-GlcNAc materials having such controllable biodegradability rates may be formulated into membranes, gels, sponges, microspheres, fibers and the like. According to a preferred embodiment of the invention, a 100% deacetylated or partially deacetylated p-GlcNAc having a
15 predictable rate of biodegradability may be utilized.

The p-GlcNAc/drug compositions of the invention may be delivered to a patient via a variety of routes using standard procedures well known to those of skill in the art. For example, such delivery may be site-specific, oral, nasal,
20 intravenous, subcutaneous, intradermal, transdermal, intramuscular or intraperitoneal administration. With respect to site-specific delivery, administration methods may include, but are not limited to injection, implantation, arthroscopic, laparoscopic or similar means. p-GlcNAc
25 membranes and/or gels as well as microspheres and sponges are preferred for such site-specific delivery methods.

As noted supra, the p-GlcNAc of the compositions of the invention may be formulated into membranes, gels, sponges, microspheres, fibers, and the like. These p-GlcNAc products
30 adhere and mold to tissues, both soft and hard tissues, in the human body with no need for suturing. The p-GlcNAc materials may, for example, be applied during general or minimally invasive surgery, such as laparoscopic surgery.

According to a preferred embodiment of the invention,
35 the p-GlcNAc is in the form of a gel in which the endothelin antagonist and/or other antitumor agent is dissolved or otherwise incorporated. p-GlcNAc-based gels and membranes

have a variety of applications as therapeutic drug delivery systems, e.g., to provide site-specific slow-release delivery directly to a tumor or to the region vacated by a tumor following surgery. Such an immobilized slow-release
5 composition can act as an important initial defensive procedure after surgery. In addition, such antitumor drug delivery systems can be particularly useful in treating tumors which are totally or partially inaccessible to surgery such as, e.g., certain brain tumors.

10 The EA/p-GlcNAc compositions of the invention are therefore useful as therapeutic drug delivery systems for the treatment of cancer and other proliferative diseases. These compositions can additionally include other antitumor agents, which can be attached to, or encapsulated within, the p-
15 GlcNAc of the invention to provide a synergistic effect. Such antitumor agents are well known to those of skill in the art, and include, but are not limited to, the following categories and specific compounds: alkylating agents, antimetabolite agents, anti-tumor antibiotics, vinca alkaloid
20 and epipodophyllotoxin agents, nitrosoureas, enzymes, synthetics, hormonal therapeutic biologics and investigational drugs.

Such alkylating agents may include, but are not limited to, nitrogen mustard, chlorambucil, cyclophosphamide,
25 ifosfamide, melphalan, thiopeta and busulfan.

Antimetabolites can include, but are not limited to, methotrexate, 5-fluorouracil, cytosine arabinoside (ara-C), 5-azacytidine, 6-mercaptopurine, 6-thioguanine, and fludarabine phosphate. Antitumor antibiotics may include but
30 are not limited to doxorubicin, daunorubicin, dactinomycin, bleomycin, mitomycin C, plicamycin, idarubicin, and mitoxantrone. Vinca alkaloids and epipodophyllotoxins may include, but are not limited to vincristine, vinblastine, vindesine, etoposide, and teniposide.

35 Nitrosoureas include carmustine, lomustine, semustine and streptozocin. Enzymes can include, but are not limited, to L-asparagine. Synthetics can include, but are not limited

to Dacrabazine, hexamethylmelamine, hydroxyurea, mitotane procabazine, cisplatin and carboplatin.

Hormonal therapeutics can include, but are not limited to corticosteroids (cortisone acetate, hydrocortisone, prednisone, prednisolone, methyl prednisolone and dexamethasone), estrogens, (diethylstilbestrol, estradiol, esterified estrogens, conjugated estrogen, chlorotiansene), progestins (medroxyprogesterone acetate, hydroxy progesterone caproate, megestrol acetate), antiestrogens (tamoxifen), aromatase inhibitors (aminoglutethimide), androgens (testosterone propionate, methyltestosterone, fluoxymesterone, testolactone), antiandrogens (flutamide), LHRH analogues (leuprolide acetate), and endocrines for prostate cancer (ketoconazole).

Biologics can include, but are not limited to interferons, interleukins, tumor necrosis factor, and biological response modifiers.

Investigational Drugs can include, but are not limited to alkylating agents such as Nimustine AZQ, BZQ, cyclodisone, DADAG, CB10-227, CY233, DABIS maleate, EDMN, Fotemustine, Hepsulfam, Hexamethylmelamine, Mafosamide, MDMS, PCNU, Spiromustine, TA-077, TCNU and Temozolomide; antimetabolites, such as acivicin, Azacytidine, 5-aza-deoxycytidine, A-TDA, Benzyldiene glucose, Carbetimer, CB3717, Deazaguanine mesylate, DODOX, Doxifluridine, DUP-785, 10-EDAM, Fazarabine, Fludarabine, MZPES, MMRP, PALA, PLAC, TCAR, TMQ, TNC-P and Piritrexim; antitumor antibodies, such as AMPAS, BWA770U, BWA773U, BWA502U, Amonafide, m-AMSA, CI-921, Datelliptium, Mitonafide, Piroxantrone, Aclarubicin, Cytorhodin, Epirubicin, esorubicin, Idarubicin, Iodo-doxorubicin, Marcellomycin, Menaril, Morpholino anthracyclines, Pirarubicin, and SM-5887; microtubule spindle inhibitors, such as Amphethinile, Navelbine, and Taxol; the alkyllysophospholipids, such as BM41-440, ET-18-OCH₃, and Hexacyclopophosphocholine; metallic compounds, such as Gallium Nitrate, CL286558, CL287110, Cycloplatam, DWA2114R, NK121, Iproplatin, Oxaliplatin, Spiroplatin, Spirogermanium, and

Titanium compounds; and novel compounds such as, for example, Aphidoicolin glycinate, Ambazone, BSO, Caracemide, DSG, Didemnin, B, DMFO, Elsamicin, Espertatrucin, Flavone acetic acid, HMBA, HHT, ICRF-187, Iododeoxyuridine, Ipomeanol, Liblomycin, Lonidamine, LY186641, MAP, MTQ, Merabarone SK&F104864, Suramin, Tallysomyacin, Teniposide, THU and WR2721; and Toremifene, Trilosane, and zindoxifene.

Antitumor drugs that are radiation enhancers are preferred for instances in which radiation therapy treatment is to be prescribed, either in lieu of, or following surgery. Examples of such drugs include, for example, the chemotherapeutic agents 5'-fluorouracil, mitomycin, cisplatin and its derivatives, taxol, doxorubicin, actinomycin, bleomycins, daunomycins, and methamycins.

Additional synergistic effects can be obtained using the EA/GlcNAc compositions of the invention in combination with two or more other antitumor agents such as thioguanine combined with cytosine arabinoside (ara-C) for the improved treatment of acute nonlymphocytic leukemia, tamoxifen with cisplatin for breast cancer, and prostaglandins with cisplatin for breast and prostate cancer. Many other synergistic combinations of anti-cancer drugs, known to those of skill in the art, may be used with the EA/p-GlcNAc and EA/p-GlcNAc derivative formulations of the invention.

Additionally, the use of the p-GlcNAc-containing compositions of the invention is desirable given that the p-GlcNAc polymer has chemical properties and characteristics making possible the formulation and delivery of some drugs that have heretofore been difficult to formulate and deliver. For example, taxol, a microtubule spindle inhibitor drug used to treat breast cancer, is hydrophobic and requires the addition of polyoxyethylated castor oil in order to solubilize it as a liquid infusion for intravenous delivery. The hydrophobic nature of taxol makes it an ideal compound for formulation with p-GlcNAc polymer materials for topical controlled release delivery. United States Patent 5,635,493 at Section 23, incorporated herein by reference, presents

such a p-GlcNAc/taxol formulation. Additional targets for p-GlcNAc antitumor systems include, but are not limited to, skin, GI tract, pancreatic, lung, breast, urinary tract and uterine tumors, and HIV-related Kaposi's sarcomas.

5 Because the p-GlcNAc materials of the invention are themselves immunoneutral, in that they do not elicit an immune response in humans, such p-GlcNAc devices, as described above, comprising p-GlcNAc membranes, 3D porous matrices and/or gels that harbor immobilized drugs, may
10 deliver such drugs in a manner such that there is no immune response. Certain additional materials, such as natural alginates and synthetic polymers, may be used in some cases to construct such devices in combination with the p-GlcNAc material. For instance, a polymeric delayed-release drug
15 delivery system can be manufactured in a manner similar to that suggested by A. Polk (Polk, A. et al., 1994, J. of Pharmaceutical Sciences, 83(2):178-185). In such a procedure, deacetylated p-GlcNAc is reacted with sodium alginate in the presence of calcium chloride to form
20 microcapsules containing the drug to be delivered and released under appropriate conditions and over a certain lapse of time.

The therapeutically effective doses of any of the drugs or agents described above, in conjunction with the p-GlcNAc-
25 based systems described herein, may be routinely determined using techniques well known to those of skill in the art. A "therapeutically effective" dose refers to that amount of the compound sufficient to result in amelioration of symptoms of the processes and/or diseases described herein.

30 Toxicity and therapeutic efficacy of the drugs can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the
35 population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit large therapeutic

indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography. According to a preferred embodiment, the dose range of the endothelin antagonists used in the compositions of the invention is from about 1 mg/kg to about 100 mg/kg.

Further, the doses of many of the antitumor drugs listed above are well known to those of skill in the art and can be easily found in such compendia as the PHYSICIANS DESK REFERENCE, Medical Economics Data Publishers; REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Co.; GOODMAN & GILMAN, THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, McGraw Hill Publishers, THE CHEMOTHERAPY SOURCE BOOK, Williams and Wilkins Publishers, online services such as the Cancer Lit®, U.S. National Cancer Institute database, as well as reports of pharmacological studies such as "A MultiCenter Randomized Trial of Trial of Two Doses of Taxol" Nabholz, J.M., Gelmon, K., Bontenbal, M. et al. Medical Education Services

Monograph - 1994 Bristol-Myers Squibb Company Publication;
"Randomized Trial of Two Doses of Taxol in Metastatic Breast
Cancer: An Interim Analysis" Nabholz, J.M., Gelmon, K.,
Bontenbal, M., et al. 1993, Proc. Am. Clin. Oncol., 12:60.
5 Abstract 42.

The dose ranges for antitumor drugs in the compositions
of the invention may be lower than, equal to or greater than
the typical daily doses prescribed for systemic treatment of
patients. For example, dosages of 5'-FU equivalent to 50% of
10 the standard dosages used to treat colorectal cancer with 5'-
FU in humans (300-450 mg/m² i.v. daily for 5 days) resulted
in an 80-90% reduction in volume of ectopic HT29 colon cancer
tumor implants in scid mice. The use of the p-GlcNAc
membrane as a drug delivery matrix for the administration of
15 5'-FU reduced the dosage required to dramatically reduce
tumor volume by 50% as compared to intravenous control
animals. Details regarding this data can be found in Example
Section 21 of United States Patent 5,635,493, which is
incorporated herein by reference. In cases where higher
20 doses are required, these higher doses may be tolerated in
that the drugs are delivered locally at the site of a tumor,
and therefore, other tissues, including blood cells, are not
as readily exposed to the drugs.

Certain antitumor agents are vesicants, including
25 dactinomycin, daunomycin, doxorubicin, estramustine,
mechlorethamine, mitomycin C, vinblastine, vincristine and
vindesine; while certain antitumor drugs are irritants,
including carmustine, decarbazine, etoposide, mithramycin,
streptozocin and teniposide. Vesicants and irritants cause
30 adverse side-effects including extravasation and irritation
of tissues with pain, redness, swelling, and other symptoms.
Further, tissue necrosis can result from some of the side
effects. The p-GlcNAc membrane and gel materials of the
compositions of the invention used for the topical,
35 controlled release of antitumor drugs have wound healing
properties. Normal tissues that are in contact with vesicant
or irritant antitumor drugs delivered by the p-GlcNAc

membrane and gel formulations of the invention are, therefore, not as readily damaged and will heal faster due to the active healing effects of the p-GlcNAc component of the p-GlcNAc-containing compositions of the invention.

5

6. EXAMPLE: PURIFICATION OF p-GlcNAc USING THE MECHANICAL FORCE PURIFICATION METHOD

In this section, p-GlcNAc was purified using the Mechanical Force technique described in Section 5.1, supra.

10

6.1 MATERIALS AND METHODS/RESULTS

Diatom culture conditions: The diatom species Thalassiosira fluviatilis was grown in culture according the procedures described in United States Patent 5,635,493, incorporated herein by reference.

15

SEM procedures: The SEM techniques used here were as follows: A Zeiss 962 instrument was utilized with an accelerating voltage of 10kv, and a working distance of 15mm. Polaroid type 55 p/n (u4) was utilized at various magnifications, as indicated. Sample coat:carbon coat (100Å) & 100Å aupd.

20

(a) Specimen preparation: For primary fixation, the culture growth medium was replaced with 2% glutaraldehyde in Eagle's DMEM without serum. Several changes were performed to ensure a complete transition from growth media to fixative. Fixation proceeded for 0.5 hours at room temperature. Cover slips were transferred to fresh vials containing 2% Glutaraldehyde in 0.1M Na Cacodylate at pH 7.2 with 0.1M Sucrose and fixed for a further 1.5 hours at room temperature.

25

30

p-GlcNAc Purification procedure: p-GlcNAc was purified from the diatom culture by utilizing the Mechanical Force technique described in Section 5.1, supra. Specifically, the p-GlcNAc fibers were separated from the diatom cell bodies by subjecting the contents of the culture to three short bursts of top speed mixing motion in a Waring blender. Total time of the three bursts was about one second. The resulting suspension was centrifuged at 3500 rpm in a Sorvall GS-4

35

fixed angle rotor, for 20 minutes at about 10°C. The supernatant was decanted, and centrifuged again; this time at 4000 rpm, in a Sorvall GS-4 fixed angle rotor for 20 minutes at about 10°C. Once again, the supernatant was decanted and centrifuged at 4000 rpm at 10° C. The final supernatant of the third centrifugation was clear, with little, if any, visible flocs floating in the liquid. The clear supernatant was decanted into a Buchner filtration unit equipped with a Supor-800 polyether sulfone filter membrane with 0.8µm pore size (Gelman, Inc.), suction was then applied and the liquid was filtered from the fiber suspension, allowing the fibers to be collected on the membrane. The collected fibers were washed with 1 liter of distilled, deionized H₂O at 70° C. When almost all of the water had been drained, fibers were washed, with suction, with 1 liter of 1 N HCl at 70°C. When most of the acid solution had been drained, the fibers were washed with 1 liter of distilled, deionized H₂O at 70°C, using suction. When most of the wash water had been drained, the fibers were washed with 1 liter of 95% ethanol at room temperature, and vacuum was applied. The filter membrane on which the white fiber membrane had been collected was then removed from the filtration unit and the membrane and its membrane support was dried in a drying oven at 58°C for 20 minutes, after which the membrane and its support was placed in a desiccator for 16 hours.

Following this purification procedure, the yield of p-GlcNAc from a 1000 ml culture was 6.85 milligrams per liter of diatom culture.

30 7. EXAMPLE: PURIFICATION OF p-GlcNAc
 USING THE BIOLOGICAL/CHEMICAL
 PURIFICATION METHOD

In this section, p-GlcNAc was purified using two of the Chemical/Biological techniques described in Section 5.1, supra. Briefly, p-GlcNAc was purified via HF treatment, in one case, and via acid treatment/neutralization in the second case.

7.1 MATERIALS AND METHODS/RESULTS

Diatom culture conditions: The diatom species *Thalassiosira fluviatilis* was grown in a culture according to the procedures described in United States Patent 5,635,493, incorporated herein by reference.

SEM procedures: The techniques utilized in this study were as described supra.

Purification procedure: First, p-GlcNAc was purified by HF treatment. Specifically, under a fume hood, 2.42 ml of a 49% (29N) HF solution was added to the diatom contents of the culture, at room temperature, for each 1000 ml of the volume of the original cell culture, resulting in a 0.07 M HF solution. The mixture was then shaken vigorously for about 30 seconds, causing persistent foam to appear over the liquid. The container was allowed to stand undisturbed for 5-6 hours to allow heavy particulates to settle. At the end of this time, a layer of foam had formed, while the liquid itself was divided into two strata: first, a narrow, very dark green layer resting on the bottom of the container below a second, much lighter colored grayish-green and murky phase which represented perhaps 85-90% of the total volume of liquid. The foam layer was carefully siphoned off, using a capillary glass tube and vacuum suction. The grayish cloudy supernatant was then siphoned off, with care being taken not to disturb the dark bottom layer, which consisted mainly of settled cell bodies, and was transferred to a separate plastic container. The grayish cloudy supernatant was allowed to stand undisturbed for an additional 16 hours. The liquid was initially almost colorless, light grey, but not transparent. After 16 hours settling time, a small amount of foam remained on top of the main body of liquid and a small amount of green matter had settled on the bottom of the container. The liquid was lighter in color, but still not transparent. The foam on top of the liquid was siphoned off as before. The main body of liquid was then carefully siphoned off, leaving behind the small amount of settled green material at the bottom of the container. The liquid

which had thus been isolated, contained the majority of the p-GlcNAc fibers and some impurities.

To remove proteins and other unwanted matter liberated by the diatoms during the preceding steps in the procedure from the fiber-containing liquid, the suspension of fibers and cell remnants was washed with sodium dodecyl sulfate (SDS). Specifically, the necessary volume of a 20% SDS solution was added to make the final concentration of the liquid 0.5% SDS by volume. The container holding the liquid was sealed, secured in a horizontal position on a shaking machine, and agitated for 24 hours at about 100 shakes a minute. Soon after shaking began, large flocs of white p-GlcNAc fibers appeared in the suspension, and a considerable amount of foam accumulated in the head space of the containers. At the end of the SDS washing, the contents of the containers were transferred to a Buchner filtration equipment provided with a Supor-800 polyether sulfone filter membrane, with 0.8 micron pore size (Gelman, Inc.). The liquid was filtered with suction, and the p-GlcNAc fibers in the liquid were collected on the filter membrane.

The p-GlcNAc fibers collected on the filter membrane were then washed further. First, the fibers were washed with hot (70° C) distilled, deionized H₂O, using three times the volume of the original suspension. With a water jet using distilled, deionized H₂O, the white fiber clumps collected on the filter membrane of the Buchner filter were transferred to a Waring blender, and the fiber clumps were disintegrated with about 10 short mixing bursts. The suspension of disintegrated fibers was transferred to a Buchner filter funnel equipped with a polyether sulfone filter membrane as described above, and the liquid was removed under suction. The collected fibers were washed with 1000 ml of hot (70°C) 1N HCl solution, and subsequently further washed with 1000 ml hot (70°C) distilled, deionized H₂O. Finally, the fibers were washed with 1000 ml 95% ethanol at room temperature, and filtered to dryness. The fiber membrane and the filter membrane supporting the fiber membrane were then dried in a

drying oven at 58°C for 20 minutes. The membrane and membrane support was then placed in a desiccator for 16 hours. The membrane was then carefully detached from the filter membrane.

5 Second, p-GlcNAc was purified by using the acid treatment/neutralization method described in Section 5.1, supra. Specifically, the p-GlcNAc was processed as described earlier in this Section, until prior to the SDS wash step, at which point the solution was neutralized to a pH of
10 approximately 7.0 by the addition of a 2.9M Tris solution. The p-GlcNAc yield from this particular purification procedure was 20.20 milligrams per liter of diatom culture, although, on average, approximately 60 milligrams per liter diatom culture are obtained. An SEM micrograph of a membrane
15 formed as a result of the acid treatment/neutralization purification procedure is shown in Figure 3.

8. EXAMPLE: p-GlcNAc DEACETYLATION

A p-GlcNAc membrane was suspended in an aqueous 50% NaOH
20 solution. The suspension was heated at 80°C for 2 hours. The resulting deacetylated membrane was dried and studied by scanning electron microscopy, as shown in Figure 5.

9. EXAMPLE: p-GlcNAc REFORMULATION

25 A p-GlcNAc membrane (16.2 mg) was dissolved in 1 ml of a dimethylacetamide solution containing 5% LiCl. The p-GlcNAc-containing solution was placed in a syringe and extruded into 50 ml of pure water to precipitate the fibers. The resulting fiber material was studied using scanning electron
30 microscopy, as shown in Figure 6:

10. EXAMPLE: PREPARATION OF AN EA/p-GlcNAc COMPOSITION OF THE INVENTION

Ro61-0612/001 (also referred to herein as "Ro61") is a
35 non-specific, non-peptide-based endothelin antagonist with the structure as depicted in Formula I, supra. Its chemical name, in salt form, is 5-Isopropyl-pyridine-2-sulfonic acid [6-(2--hydroxy-ethoxy)-5-(2-methoxy-phenoxy), -2-[2-(1H-

tetrazol-5-yl)-pyridin-4-yl]-pyrimidin-4-yl]amide sodium salt (1:2) and its molecular weight is 649.59. Its solubility in water is greater than 3%. Ro61's binding inhibitory potency (IC_{50}) as to the ETA receptor is 1-20 nM and as to the ETB receptor is 20-30 nM. Its functional inhibitory potency (pA₂) with respect to the ETA receptor is 9.5 and with respect to the ETB receptor is 7.7. Its recommended dose in vivo is 1-30 mg/kg iv or ip. Its recommended dose in vitro is 10^{-9} to 10^{-5} M.

Ro61 for use in a composition of the invention was provided initially as a lyophilized powder from Acetelion Ltd, Allschwil, Switzerland, which was suspended in sterile water, and the pH was adjusted to 4.0 with sterile hydrochloric acid. Alternatively, Ro61 can be synthesized using techniques known in the art.

A p-GlcNAc fiber slurry was prepared as follows: p-GlcNAc prepared by the biological/chemical method described in Example Section 7 supra was resuspended in distilled-deionized water and agitated to form a fibrous suspension or slurry of about 1 mg/ml. The fiber slurry was then oven dried at 60 °C for 2 h to form p-GlcNAc polymer membranes. The membranes were deacetylated in 40% NaOH solution at 80 °C for 2 h. When the membranes reached 100% deacetylation, they were washed with distilled-deionized water until a pH of 7.0 was achieved.

The washed deacetylated membranes were then converted to p-GlcNAc lactate salt in the presence of lactic acid essentially as described in United States Patent 5,623,064 incorporated herein by reference. Briefly, the deacetylated p-GlcNAc was suspended in an organic medium such as 2-propanol (containing 10% water) so as to wet all of the deacetylated p-GlcNAc material. With stirring, an appropriate amount of 50% aqueous lactic acid solution was added. The lactic acid should be reagent grade, and must be analyzed to determine the exact concentration of available (i.e., non-esterified) lactic acid present. This was generally accomplished by titration with 0.1N NaOH to the

phenolphthalein end-point (pH 7.0). The mixture was allowed to stir for at least two hours at room temperature. Low heat may be added to increase the reaction rate. Reaction time may be extended or the amount of 50% aqueous lactic acid may be increased to ensure that the reaction goes to completion. The suspension was then finely filtered through a Buchner funnel using quantitative ashless filter paper and the material, in the form of a membrane, was washed with anhydrous 2-propanol. The membrane was then allowed to air dry in a fume hood for 2 hours and then placed in an oven at 40 °C overnight.

An EA/p-GlcNAc gel was next prepared for injection by dissolving the p-GlcNAc lactate membranes in distilled-deionized water to the desired concentration, e.g., 2% p-GlcNAc-lactate by weight and adding Ro61 to the solution. The final concentration of Ro61 in the gel was adjusted so that each animal received 3 mg/kg in a 200 µl sample of gel. Optionally, a reagent grade propylene glycol (2-propanediol) can be added to the p-GlcNAc solution to a final propylene glycol concentration of between 1-10%. In some cases, a preservative may be added to prevent bacterial and/or fungal contamination. According to other embodiments, concentrations of p-GlcNAc-lactate ranging from 0.1% to 4.0% can be prepared as described above. The viscosity of these preparations increases as the p-GlcNAc-lactate percentage increases, such that formulations having 0.5% or more of the p-GlcNAc-lactate behave as gels.

11A. EXAMPLE: B16 AS AN ENDOTHELIN RESPONSIVE TUMOR MODEL

B16 cells were evaluated for use as an endothelin responsive tumor model system. The B16 cells, i.e., from the B16 murine melanoma cell line (of fibroblastic origin), were obtained from the American Type Culture Collection (Rockville MD) as a frozen stock. The cells were cultured in complete medium (CM): RPMI 1640 (Irvine Scientific, Santa Ana CA) supplemented with 10% heat-inactivated fetal bovine serum (Summit Biotechnologies, Ft. Collins CO), penicillin (50

units/ml, streptomycin (50 μ g/ml), 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids (Gibco BRL, Gaithersburg MD), 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol (Sigma Immunochemicals, St. Louis MO). The cells were grown at 37
5 °C in a humidified 5% CO₂ incubator and adjusted to 1 X 10⁵ cells/ml every second day.

The B16 cells were analyzed for endothelin levels and endothelin receptor (ETR) expression as follows: ET1 in the B16 culture supernatants was measured by a competitive
10 radioimmunoassay (RPA 545, Amersham, Milford Ma) with radioactive ligand and an ET1-specific antibody. Bound and free ET were reacted with a second antibody phase system followed by magnetic separation. A standard curve was determined by calculating the percentage of bound ligand/zero
15 standard (B/Bo), and the concentration of ET could be read from this standard curve. The recovery from the extraction procedure was 75 \pm 5% based upon plasma spiked standards (4-20 fmol/ml). The interassay variation was 10% and the intra-assay variation was 9% for the ET radioimmunoassay procedure.

20 Using this assay, in one experiment, baseline ET1 levels in 24 h B16 culture supernatants were found to be 1.269 fmol/ml. Addition to the cultures of 10 μ M of either an ETA or ETB agonist or both induced increased proliferation of the B16 cells by 137%, 117% and 164% of untreated controls,
25 respectively, with corresponding ET1 levels of 34.01, 1.158, and 34.01 fmol/ml, respectively. In a subsequent experiment, the baseline levels of ET1 in 24 h B16 culture were 597 \pm 58 fmol/l and addition of 10 μ M of a non-selective agonist (ET1), a selective ETB receptor agonist (BQ3020), or both
30 increased proliferation of the B16 cells compared to untreated controls by 154%, 116% and 141%, respectively (see Table 1, below). These data indicate that murine B16 melanoma cells express ETRs, which are responsive to established endothelin agonists.

35

Table 1

	Ligand (Receptor)	% proliferation compared to untreated controls
Agonists	ET1 (ETA>ETB)	154 (\pm 20.9)
	BQ 3020 (ETB)	116 (\pm 0.8)
	ET1 + BQ 3020	141 (\pm 4.1)

5
10 In addition, the B16 cells were determined to express endothelin receptors via immunofluorescence staining using the intracellular flow cytometry kit from Pharmingen with anti-cytoplasmic endothelin receptor antibodies, i.e., directed against the cytoplasmic region of the receptor
15 (Research Diagnostics Inc., Flanders NJ) and sheep anti-mouse IgG isotypic antibody controls (Sigma Immunochemicals, St. Louis MO). According to this procedure, the B16 cells were washed in Cytofix/Cytoperm buffer (from the Pharmingen kit) and incubated for 20 min with a permeabilization solution
20 (0.1% Triton X100 in 0.1% sodium citrate). Cells were then incubated at 4 °C in the dark for 30 min with primary anti-endothelin receptor or isotype control antibodies, and then washed and incubated an additional 30 min with the secondary FITC-labeled antibody (mouse anti-sheep IgG; Sigma). Cells
25 were visualized and photographed using a Axioplan research microscope (Carl Zeiss Inc. Jena Germany) equipped with a 100 watt mercury light source and a 40x plan-neufluar nal.3 objective.

30 These immunofluorescence staining experiments showed that B16 melanoma cells expressed detectable levels of ETA and ETB receptors.

Binding assays with ET-1¹²⁵ also suggested that B16 cells express ET receptors. These assays were performed as follows: Aortic vascular smooth muscle cells termed A10
35 cells, B16 and CHO cells were suspended at a concentration of 2×10^6 cells/ml in binding buffer (50 mM Tris/HCl - pH = 7.4, 5 mM EDTA, and 0.5% BSA) in individual tubes. The A10 were

utilized as an ETA positive control, whereas CHO cells were utilized as a negative control, demonstrating no binding of labeled ET1, consistent with the absence of ETR on this cell line.

5 An aliquot of 21 μ l of (3-[125 I] iodotyrosyl) endothelin-1 (ET1-I 125 , from Amersham Life Science Inc, Arlington Heights, IL) was added to each tube at a final concentration of 10^{-12} M. Labeled cells were then aliquoted into microcentrifuge tubes and 18 μ l of unlabeled ET1 (at a final concentration of
10 10^{-8} M) or HBSS was added. The tubes were mixed and each sample was divided into 300 p1 aliquots, placed in siliconized tubes and shaken for 2.5 hours at 37°C. After incubation, tubes were centrifuged at 10,000 rpm for 6 minutes, cell pellets resuspended by vortexing in 300 μ l of
15 binding buffer and washed twice again in binding buffer. After the final wash, cells were resuspended in 500 μ l 1N NaOH, shaken for 10 minutes at 37°C and placed into scintillation tubes for counting on a Packard Cobra Auto-gamma 5000 Series (Model 5002) gamma counting system (Packard
20 Instrument Co., Meridea, CT).

The results of these ET1 binding assays, demonstrating that B16 cells express ETR, are shown in Table 2 below.

Table 2

25 Binding of ET1 to B16 tumor cells.

	A10	B16	CHO
ET1 binding*	53885 (\pm 2555)	25605 (\pm 3801)	119 (\pm 91)
30 ET1 binding with competitor**	1135 (\pm 120)	1398 (\pm 691)	346 (\pm 259)

Results are expressed as mean cpm per 10^6 cells (\pm SEM).

* ET1-I 125 was added at a final concentration of 10^{-12} M.

35 ** Competitor was unlabeled ET1 added at a final concentration of 10^{-8} M.

11B. EXAMPLE: Ro61 INHIBITION OF MELANOMA
CELL PROLIFERATION IN VITRO

Normal splenocytes do not show visible levels of ETR as detected by immunofluorescence as described above. Due to the facility of splenocyte isolation and culture, they were therefore used as control cells to assess the effects of Ro61 on cell proliferation. Splenocytes were harvested from female 6-8 week old C57BL/6 (H-2b) mice (Jackson Laboratories, Bar Harbor MA) as follows: spleens were removed, placed in CM, and their cells dispersed with a 3 cc syringe plunger. The cell suspension was then filtered through a 70 μ m cell strainer and erythrocytes were lysed with ammonium chloride lysis solution (prepared by mixing 9 parts 8.3 g/L ammonium chloride; 1 part 20.59 g/L Tris, pH 7.65 immediately before use). The splenocytes were then washed and resuspended in CM.

Ro61 was obtained as a lyophilized powder from Acetelion Ltd, Allschwil, Switzerland as described above and proliferation assays were performed to assess the effect of Ro61 on B16 cell proliferation in vitro. More specifically, Ro61 in HBSS was added at increasing concentrations to a 96 well culture plate. B16 cells or control splenocytes as described above were then added to the wells at a concentration of 10^5 cells per well and grown for 72 h. Cell proliferation was assayed using the CellTiter 96 kit for non-radioactive cell proliferation measurement (Promega, Madison WI) as follows: Briefly, cells were incubated with 15 ml of MTT dye (from Promega kit) for 4 h after which formazan crystal formation was visible. Crystals were dissolved in 50 ml of solubilization/stop solution (from Promega kit) for 30 min at room temperature and color changes were measured as OD 570 nm. Background OD at 630 was subtracted automatically. Mean values of triplicate wells were determined. Cell proliferation or death was also recorded by photography of light microscopy at 40X focal power.

The inhibitory effect of Ro61 on the B16 melanoma cells is depicted in Figure 7. Proliferation of Ro61-treated cells is expressed as a percentage of untreated control cells.

Mean values of triplicate wells were determined. As can be seen in Figure 7, Ro61 inhibited the proliferation of the B16 cells (closed circles) but did not inhibit the normal splenocytes (open circles). Moreover, when Ro61 was added at increasing concentrations to the cells in culture, a dose dependent inhibition was observed. This effect was seen at concentrations of 0.1 μ M (22% inhibition compared to untreated controls) and was maximal at 10 μ M (83% inhibition). Microscopically, at the highest concentration of Ro61, the B16 cells no longer had a normal fibroblast-like spindle shape but were round and sparse with poor viability (see FIGS. 17A-17B). In contrast to this, the splenocytes were only minimally affected by the addition of even the highest concentration of Ro61 (10 μ M).

It was also found that endothelin levels in the B16 culture supernatants increased (from 513 ± 27 fmol/l at 50 nM of Ro 61 to 954 ± 31 fmol/l at 5 μ M of Ro 61) in a dose-dependent manner ($p < 0.001$). This observation is consistent with Ro61 blockade of ETR, resulting in the interruption of a receptor-mediated feedback loop.

Since Ro61, an inhibitor of the ET receptor, caused the inhibition of B16 cell proliferation, it was of interest to determine whether the addition of peptide agonists specific for ETA or ETB could reverse this effect, i.e., by competing with Ro61 for receptor binding sites. The agonist, BQ-3020-[Ac-[Ala^{11,15}]-endothelin(6,21), i.e., BQ3020 (Novabiochem, catalog no. A1 4534) and the agonist, [Ala^{1,3,11,15}]-endothelin¹ (Sigma Immunochemicals, St. Louis MO, catalog no.: E6877) were suspended in HBSS for use. 5×10^4 B16 cells were cultured with either agonist alone, both agonists together or neither at a concentration of 10 nM in each well and Ro61 was then added at various concentrations to the wells.

As demonstrated in Figure 10, Ro61 inhibition of B16 proliferation was prevented by adding ET receptor agonists. Proliferation of Ro61-treated cells is expressed as a percentage of untreated control cells. Mean values of triplicate wells were determined. First, as indicated by the

Y axis of Figure 10 (0 concentration of Ro61), the addition of the BQ3020 agonist (closed triangle) or the ET-1 agonist (open diamond) alone, or the two agonists in combination (open box), induced the proliferation of the B16 cells. For the BQ3020 agonist, proliferation was 137% of untreated controls, for the ET1 agonist, the proliferation was 117% of untreated controls and for the combination of agonists, the proliferation was 164% of untreated controls.

As indicated further in Figure 10, the addition of these agonists also counteracted the inhibition induced by increasing doses of Ro61. For example, the addition of ET1 at a concentration of 10 nM completely reversed the effects of Ro61 at a concentration of 1 nM and decreased the inhibition of Ro61 at 5 μ M by 50%. The BQ3020 agonist was able to significantly reverse the effects of 5 μ M Ro61 (only 12% inhibition of proliferation at 5 μ M). The most significant effect was observed with the combination of the two agonists inducing proliferation (123% of untreated controls) even with addition of 0.1 μ M Ro61. These dose dependent findings defined an endothelin receptor-mediated proliferation response for the murine B16 cells that can be inhibited by an ETR antagonist.

In other experiments, Ro61, an antagonist of both ETA and ETB having an approximately 10 fold higher affinity for ETA, was tested along with BQ123, an ETA antagonist and BQ788, an ETB antagonist (both obtained from the American Peptide Co., Sunnyvale, CA) to determine the effect of these antagonists on B16 cell proliferation using the cell proliferation assay described supra. The presence of BQ123, BQ788, BQ123 + BQ788, or Ro 61-0612/001 (each at a total concentration of 10 μ M) in culture resulted in significant inhibition of proliferation (16, 18, 19 and 50%, respectively, compared to non-treated controls; $p = 0.001$, see Table 3 below).

35

Table 3

	Ligand (Receptor)	% proliferation compared to untreated controls
Antagonists	BQ 123 (ETA)	84 (\pm 6.6)
	BQ 788 (ETB)	82 (\pm 1.9)
	BQ123 + BQ 788	81 (\pm 4.5)
	Ro 61 (ETA>ETB)	50 (\pm 4.8)

In yet other proliferation assays as described supra, various endothelin antagonists were tested to determine their effect on B16 cell proliferation. The B16 cells, which had been passaged ten times in animals and express a wild type full length ETR, were compared to control B16 cells, designated FO, which express a truncated ETA mRNA and thus appear to produce an incomplete ETA receptor.

As depicted in Figures 8 and 9, the endothelin antagonists, IRL, BQ123, BQ610, BQ485, BQ788, RES, at 10^{-6} M concentration, all inhibited B16 cell proliferation compared to the FO controls and the endothelin agonist controls, ET1 and BQ3020.

12. EXAMPLE: Ro61 INDUCTION OF B16 MELANOMA TUMOR APOPTOTIC CELL DEATH

In vitro studies indicated that Ro61 treatment not only contributed to an inhibition of cell proliferation but that there was a significant component of cell death or necrosis present. In fact, B16 cells treated with Ro61 showed a significant degree of morphological changes consistent with programmed cell death (see Figure 11).

Therefore, the effect of Ro61 on B16 apoptotic cell death was investigated. B16 cells were grown in CM in 25 ml culture flasks, with or without 1 μ M Ro61, at 37 °C in a 5% CO₂ incubator for up to 72 h. Cells were assayed for apoptosis or cell death using the Fluorescein In Situ Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany) as follows: Briefly, cells were trypsinized and washed with

PBS containing 1% bovine serum albumin (BSA). After fixing with a 4% paraformaldehyde solution in PBS (pH 7.4) for 30 min, cells were permeabilized with a solution of 0.1% Triton X100 in 0.1% sodium citrate for 2 min on ice. Cells were
5 then washed and labeled with TUNEL reaction mixture (Boehringer kit) for 1 h at 37 °C and washed. Fluorescence was analyzed on a Coulter EPICS XL flow cytometer (Coulter, Miami FL). Measurements were compared to a positive control using 100 µg/ml of DNase I (Boehringer Mannheim, Mannheim,
10 Germany) for 10 min at room temperature to induce double stranded DNA breaks.

As depicted in Figure 12, the endothelin antagonist Ro61 induced apoptosis in B16 melanoma cells in culture. For example, the addition of 1 µM of Ro61 to the B16 cells
15 significantly increased the percentage of cells undergoing apoptosis as compared to untreated controls ($p = 0.0007$). The increase in percentage of cells positive by the above-described TUNEL assay was detectable as early as 24 h and was still detectable at 72 h. The effect of duration after
20 culture with Ro61 was not significant; however, the increased apoptosis of Ro61-treated cells compared to the untreated controls was highly significant (12.7%, 95% confidence interval: 11.7%-13.8%). These results established the presence of apoptotic cell signaling mediated through the ETR
25 which can be induced by an ETR antagonist. Thus, apoptosis is contributing at least in part to the inhibition of cellular proliferation and the cell death observed.

30 13. EXAMPLE: INHIBITION OF B16 MELANOMA INTRAPERITONEAL CARCINOMATOSIS IN VIVO BY AN ENDOTHELIN ANTAGONIST OR AN EA/p-GlcNAc COMPOSITION OF THE INVENTION

The striking effects of Ro61 on B16 cells in vitro led to further studies to evaluate the impact of ETR antagonism on in vivo tumor growth utilizing an aggressive intraperitoneal (IP) B16 melanoma metastases/carcinomatosis
35 model. According to this model, female C57BL/6 mice were injected intraperitoneally with 5×10^4 B16 cells in 100 µl HBSS. One day later, the mice were injected with 100 µl of

either HBSS alone (no treatment), HBSS containing 3 mg/kg of Ro61 (administered daily), HBSS containing 30 mg/kg of Ro61 (administered daily), p-GlcNAc gel (2%) alone, or p-GlcNAc gel (2%) containing 18 mg/kg of Ro61.

5 Animals in the groups treated with HBSS containing either 3 mg/kg or 30 mg/kg of Ro61 alone received daily i.p. injections while all of the other groups were treated only once. The p-GlcNAc gel alone that was utilized in this experiment was prepared as indicated in Example Section 10,
10 supra, minus addition of the Ro61. The Ro61/p-GlcNAc utilized in this experiment was also prepared as detailed above in Section 10. The mice were sacrificed after 7 days and evaluated the presence of intraperitoneal disease. More specifically, individual tumor colonies were counted under a
15 dissecting microscope for each animal on the peritoneal surface, mesentery, liver, spleen, and pancreas. Studies were conducted under double blind conditions. Photographs of the peritoneum and digestive organs were taken as well as sections of the mesentery, liver, spleen and pancreatic fat
20 and peritoneum for histological analysis by H and E as well as melanin staining.

Figure 13A shows that those mice that received daily IP injections of Ro61 at a low dose, e.g., 3 mg/kg for 6 days (for a total of 18 mg/kg) did not exhibit significantly lower
25 numbers of metastases or tumor colonies per animal. (As used herein, the term metastasis refers to tumor colonies detected in the peritoneum and digestive organs of mice injected with B16 melanoma cells according to the carcinomatosis model described above).

30 In contrast, daily injections of higher doses of Ro61 alone at 30 mg/kg for 6 days (for a total dose of 180 mg/kg) did significantly decrease the mean number of tumor colonies on day 7 after tumor injection.

In addition, as depicted in Figure 13B, the p-GlcNAc gel
35 alone reduced the mean number of tumor colonies compared to untreated controls when it was injected IP but did not have any significant effect on colony growth when injected

subcutaneously (SC). Figure 13B also shows that the greatest reduction in tumor colonies occurred with the administration of a single dose (18 mg/kg) of Ro61 formulated in the p-GlcNAc gel (mean number of colonies = 2.7 ± 1.4). The gel/Ro61 composition resulted in significantly fewer colonies when compared to both high dose Ro61 ($p=0.001$) and p-GlcNAc gel alone IP ($p=0.02$). Low dose Ro61 treatment and p-GlcNAc alone SC were not significantly different from untreated controls.

To determine whether Ro61 has not only a local effect due to p-GlcNAc but also a systemic effect, we investigated the effects of IP Ro61 treatment in a subcutaneous, SC, model of B16 melanoma. Female CS7BL/6 mice were injected SC in the flank with 5×10^4 B16 melanoma cells in 100 μ l HBSS. After 24 hours, the animals received one of the following treatments (100 μ l, IP): HBSS (daily $\times 6$), 30 mg/kg Ro61 in HBSS (daily $\times 6$), p-GlcNAc gel (one administration), or 18 mg/kg Ro61 in p-GlcNAc gel (one administration). Animals were monitored for tumor appearance and growth for a period of 3 weeks after tumor injection ($n=10$ in all groups).

As demonstrated in Figure 14, the SC model of B16 melanoma showed that all animals receiving IP Ro61 in HBSS developed tumors by day 17; however, there was a significant delay in tumor appearance (50% tumor bearing mice at day 15 compared to 100% tumor bearing mice at day 15 in the untreated controls). The combination of Ro61 plus pGlcNAc resulted in a delay in tumor appearance (50% tumor bearing mice at day 13) as well as 10% tumor free animals at 21 days post tumor injection, suggesting that sustained release of Ro61, in a separate compartment from the tumor, can significantly affect B16 melanoma growth. There were no differences in tumor appearance and growth between the untreated control group and the IP p-GlcNAc group.

Using the carcinomatosis model described supra, the effect of various other endothelin antagonists on B16 tumor colony formation was evaluated. In these experiments, the animals were injected with 100 μ l of samples containing 14.3

mg/ml of each drug or drug mixture in a single dose. As demonstrated in Figure 15, a mixture of BQ123 (an ETA antagonist) and BQ788 (an ETB antagonist) at a final total concentration equal to that of Ro61 (an ETA and ETB antagonist) significantly decreased the number of tumor colonies to a degree similar to that demonstrated by Ro61, as compared with untreated control B16 cells. Furthermore, when the BQ mixture was combined with p-GlcNAc as described supra, the number of tumor colonies were further decreased and to a degree almost equivalent to that seen with Ro61 plus p-GlcNAc. Finally, the mixed ETA/ETB antagonist, GRGDS, a commercially available pentapeptide (American Peptide Co.), when combined with p-GlcNAc, demonstrated an even greater decrease in tumor colony number than Ro61/p-GlcNAc (see Figure 15, last column).

14. EXAMPLE: LONG TERM SURVIVAL OF
C57BL/6 MICE AFTER INTRAPERITONEAL
B16 MELANOMA CHALLENGE WITH AN
ENDOTHELIN ANTAGONIST ALONE OR AN
EA/p-GlcNAc COMPOSITION OF THE INVENTION

We also evaluated endothelin antagonism therapy in long term survival experiments in vivo. The results are shown in Figure 16. Female C57BL/6 mice were injected intraperitoneally with 5×10^4 B16 cells in 100 ml HBSS. Animals were randomly separated into 4 groups for either of the following treatments: (a) no treatment (closed boxes); (b) 100 μ l of p-GlcNAc gel alone (crosses); (c) 100 μ l of daily HBSS containing 3 mg/kg Ro61 (closed triangles); or (d) 100 μ l of p-GlcNAc gel containing 3 mg/kg Ro61 (open boxes). Animals were monitored daily and sacrificed for humane reasons when determined moribund.

It is of interest to note that the B16 melanoma is an extremely virulent tumor, which results in a 0% survival rate consistently within 19-20 days of tumor injection. As indicated in Figure 10, the injection of p-GlcNAc alone delayed death by 5 days but did not increase the survival rate of the animals. However, the combination of p-GlcNAc and Ro61 at a low dose (3 mg/kg) also delayed death and 33%

of the animals showed no evidence of tumor at day 33 after tumor injection. Daily injections of the same low dose of Ro61 alone did not affect survival of the mice.

15. DISCUSSION OF RESULTS

5 The studies conducted herein show direct evidence that inhibiting the binding of endothelins to their receptors can affect the normal proliferation of a murine melanoma cell line, both in vitro and in vivo. For example, the endothelin antagonist, Ro61, is an inhibitor of both the ETA and ETB
10 receptors with an approximately 10-fold higher affinity for ETA. This correlates well with the dose dependent inhibition of melanoma cell proliferation by Ro61 in our experiments, as well as the stronger countering effect to this inhibition obtained by addition of an ETA agonist as opposed to an ETB
15 agonist. In addition, numerous other endothelin antagonists have also been demonstrated herein as inhibiting melanoma cell proliferation, including both ETA and ETB-specific antagonists, e.g., BQ123, BQ485, BQ610, BQ788, RES and IRL.

20 It is interesting to note that melanoma cells have been shown to express high levels of ET receptors and are more susceptible to endothelin antagonist inhibition than normal splenocytes, known to have much lower membrane ET receptors. Thus, the evidence presented herein points to endothelin antagonists such as Ro61, BQ123, BQ485, BQ610, BQ788, RES and
25 IRL as having an anti-proliferative effect on tumor cells in culture, which inhibition of tumor cell growth is mediated by binding to endothelin receptors that respond to peptide agonists specific for ETA and/or ETB.

30 In addition, our studies indicate that endothelin antagonists alone or in combination with the p-GlcNAc described herein, significantly reduce carcinomatosis in vivo. Moreover, the EA/p-GlcNAc compositions of this invention, e.g., Ro61/p-GlcNAc, dramatically increase the survival rate of tumor cell-bearing animals in vivo. The
35 effect of Ro61 appears to involve an apoptotic mechanism of action, which does not contradict some of the known

mechanisms of action of endothelins and their signal transduction mechanisms.

Pharmacokinetic studies with Ro61 indicate that it is metabolized relatively quickly in vivo, e.g., within 2-4 hours; however, when Ro61 is combined with the p-GlcNAc polymer of this invention, the Ro61 is retained in the gel and released more slowly, and can be detected, e.g., for at least 48 hours. Furthermore, if the concentration of the endothelin antagonist in the gel is increased, the antagonist can be retained in the gel for at least 72 hours (data not shown). Thus, by modifying the concentration of endothelin antagonist in the p-GlcNAc of the invention, one can obtain a desired slow release, and thus an enhanced effect, of the antagonist in treating proliferative disease.

Finally, as indicated in Table 4 below, other cell types that express the ETA receptor also react to the endothelin antagonist Ro61 as described herein, i.e., to exhibit an inhibition of cell proliferation upon exposure to the endothelin antagonist. This data correlates, in different cell types, the presence of, e.g., the ETA receptor with the effect of endothelin antagonists on cell proliferation. For example, these results indicate that if a tumor such as a pancreatic, breast or prostate tumor, expresses the ETA receptor, it can be treated with an ETA-sensitive endothelin antagonist as disclosed herein.

Table 4

		ETA	Ro61 effect
negative control	<i>CHO</i>	NO	NO (5/5)
melanocyte melanoma	<i>melanocyte</i>	YES	YES (2/2)
	<i>B16 F10</i>	YES	YES (5/5)
	<i>MEL 501</i>	NO	NO
	<i>MEL 888</i>	YES	YES (2/3)
pancreatic cancer	<i>ASPC 1</i>	YES	YES (5/5)
	<i>ASPC4</i>	NO	NO (5/5)
	<i>CAPAN 1</i>	NO	NO (4/5)
	<i>PANC 1</i>	YES	YES (4/5)
	<i>BXPC3</i>	YES	YES (3/4)
breast cancer	<i>MDA 231</i>	NO	NO (3/3)
	<i>MDA 453</i>	YES	YES (3/3)
prostate cancer	<i>DUI 45</i>	NO	NO (5/5)
	<i>LN CAP</i>	NO	NO (5/5)

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various
5 modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

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We claim:

1. An antitumor composition comprising at least one endothelin antagonist in combination with a poly- β -1-4-N-acetylglucosamine, said poly- β -1-4-N-acetylglucosamine
5 comprising about 4,000 to about 150,000 N-acetylglucosamine monosaccharides covalently attached in a β -1-4 conformation free of protein, substantially free of other organic or inorganic contaminants, and having a molecular weight of about 800,000 daltons to about 30 million daltons.
- 10 2. The composition of claim 1 wherein the endothelin antagonist is a non-specific endothelin antagonist.
3. The composition of claim 1 wherein the endothelin
15 antagonist is an ETA-specific endothelin antagonist.
4. The composition of claim 1 wherein the endothelin antagonist is an ETB-specific endothelin antagonist.
- 20 5. The composition of claim 1 wherein the endothelin antagonist is a peptide-based endothelin antagonist.
6. The composition of claim 1 wherein the endothelin antagonist is a non-peptide-based endothelin antagonist.
- 25 7. The composition of claim 6 wherein the non-peptide-based endothelin antagonist is a pyrimidyl sulfonamide compound.
8. The composition of claim 7 wherein the pyrimidyl
30 sulfonamide compound is Ro61.
9. The composition of claim 1 wherein the poly- β -1-4-N-acetylglucosamine comprises a poly- β -1-4-N-acetylglucosamine derivative wherein at least one N-acetylglucosamine
35 monosaccharide has been deacetylated.

10. The composition of claim 9 wherein the deacetylated monosaccharide is derivatized to a lactate salt.

11. The composition of claim 9 wherein at least about 25% to
5 about 75% of the N-acetylglucosamine monosaccharides have been deacetylated.

12. The composition of claim 1, 9 or 10 wherein the poly- β -1-4-N-acetylglucosamine is formulated as a gel.

10 13. The composition of claim 1 or 9 wherein the poly- β -1-4-N-acetylglucosamine is a mat, string, rope, membrane, fiber or sponge.

15 14. The composition of claim 12 wherein the endothelin antagonist is dissolved in the poly- β -1-4-N-acetylglucosamine gel.

15 15. An antitumor composition comprising at least one endothelin antagonist in combination with a poly- β -1-4-glucosamine, said poly- β -1-4-glucosamine comprising about 4,000 to about 150,000 glucosamine monosaccharides covalently attached in a β -1-4 conformation free of protein, substantially free of other organic or inorganic
25 contaminants, and having a molecular weight of about 640,000 daltons to about 24 million daltons.

16. The composition of claim 15 wherein the endothelin antagonist is a non-specific endothelin antagonist.

30 17. The composition of claim 15 wherein the endothelin antagonist is an ETA-specific endothelin antagonist.

18. The composition of claim 15 wherein the endothelin
35 antagonist is an ETB-specific endothelin antagonist.

19. The composition of claim 15 wherein the endothelin antagonist is a peptide-based endothelin antagonist.
- 5 20. The composition of claim 15 wherein the endothelin antagonist is a non-peptide-based endothelin antagonist.
21. The composition of claim 20 wherein the non-peptide-based endothelin antagonist is a pyrimidyl sulfonamide
10 compound.
22. The composition of claim 21 wherein the pyrimidyl sulfonamide compound is Ro61.
- 15 23. The composition of claim 15 wherein the poly- β -1-4-glucosamine is derivatized to a lactate salt.
24. The composition of claim 15 or 23 wherein the poly- β -1-4-glucosamine is formulated as a gel.
- 20 25. The composition of claim 15 wherein the poly- β -1-4-glucosamine is a mat, string, rope, membrane, fiber or sponge.
- 25 26. The composition of claim 24 wherein the endothelin antagonist is dissolved in the poly- β -1-4-glucosamine gel.
27. The composition of claim 26 wherein the endothelin antagonist is Ro61 and the poly- β -1-4-glucosamine gel is a 2%
30 gel.
28. A method for treating proliferative disease comprising administering to a patient a therapeutically effective amount of at least one endothelin antagonist in combination with a
35 poly- β -1-4-N-acetylglucosamine, said poly- β -1-4-N-acetylglucosamine comprising about 4,000 to about 150,000 N-acetylglucosamine monosaccharides covalently attached in a β -

1-4 conformation free of protein, substantially free of other organic or inorganic contaminants, and having a molecular weight of about 800,000 daltons to about 30 million daltons.

5 29. A method for treating proliferative disease comprising administering to a patient a therapeutically effective amount of at least one endothelin antagonist in combination with a poly- β -1-4-glucosamine, said poly- β -1-4-glucosamine comprising about 4,000 to about 150,000 glucosamine
10 monosaccharides covalently attached in a β -1-4 conformation free of protein, substantially free of other organic or inorganic contaminants, and having a molecular weight of about 640,000 daltons to about 24 million daltons.

15 30. The method of claim 28 or 29, wherein the proliferative disease is cancer.

31. A pharmaceutical composition for the treatment of proliferative disease comprising a therapeutically effective
20 amount of a non-peptide-based endothelin antagonist in a pharmaceutically acceptable carrier.

32. The composition of claim 31, wherein the endothelin
25 antagonist is a pyrimidyl sulfonamide compound.

33. The composition of claim 31, wherein the proliferative disease is cancer.

30 34. A method for treating cancer comprising administering to a patient a therapeutically effective amount of a non-peptide-based endothelin antagonist in combination with a pharmaceutically acceptable carrier.

35 35. The method of claim 34, wherein the endothelin antagonist is a pyrimidyl sulfonamide compound.

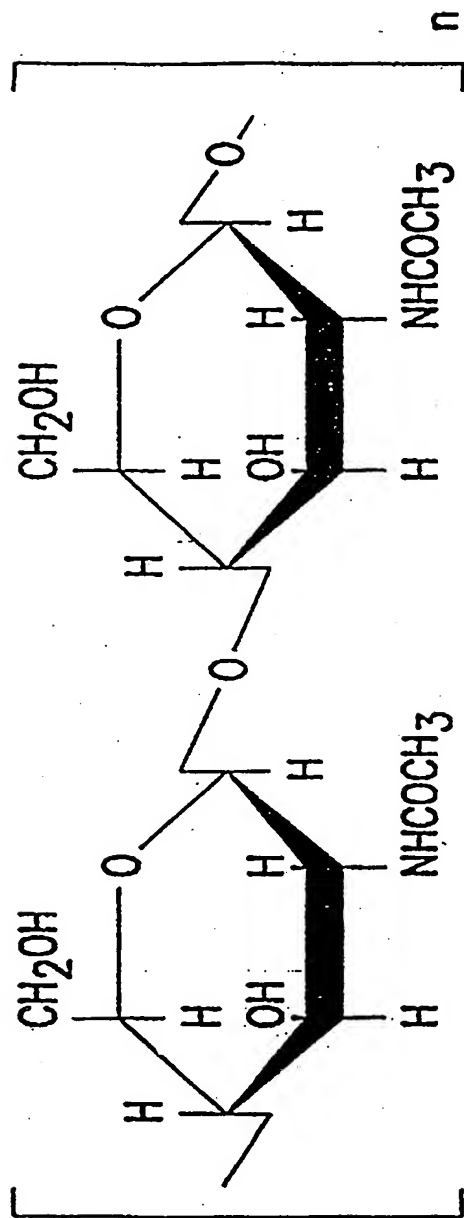


FIG. 1

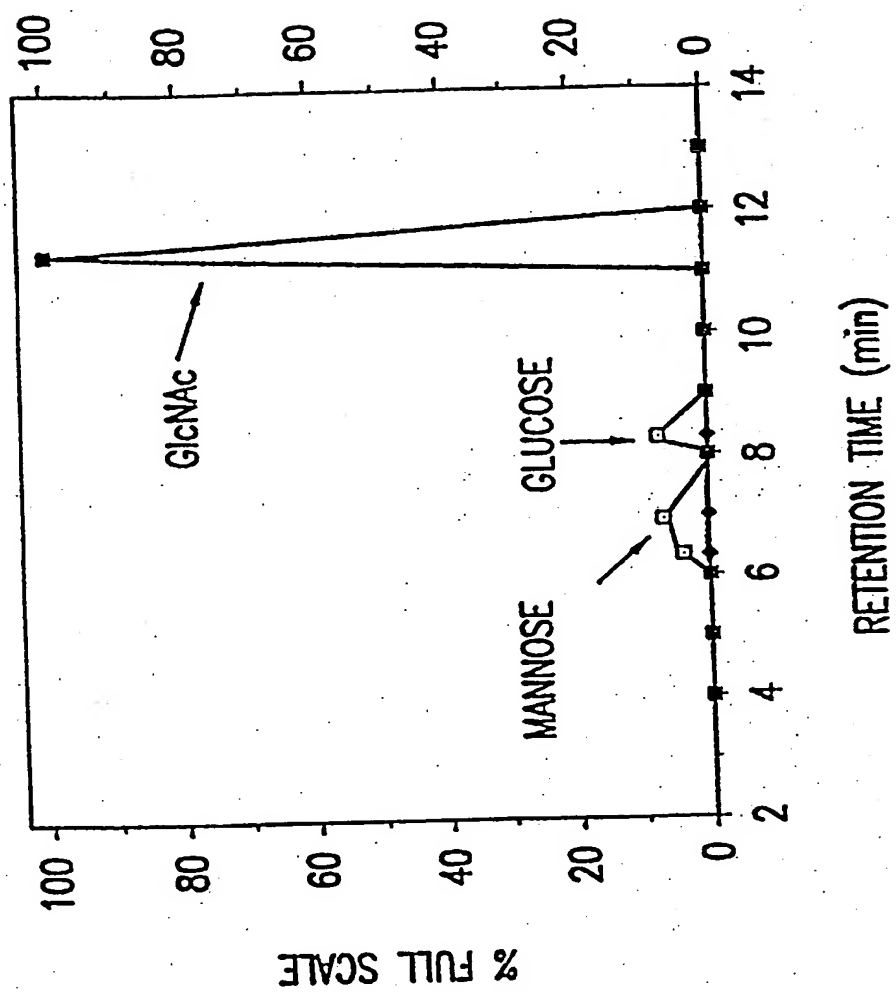


FIG. 2



FIG. 3

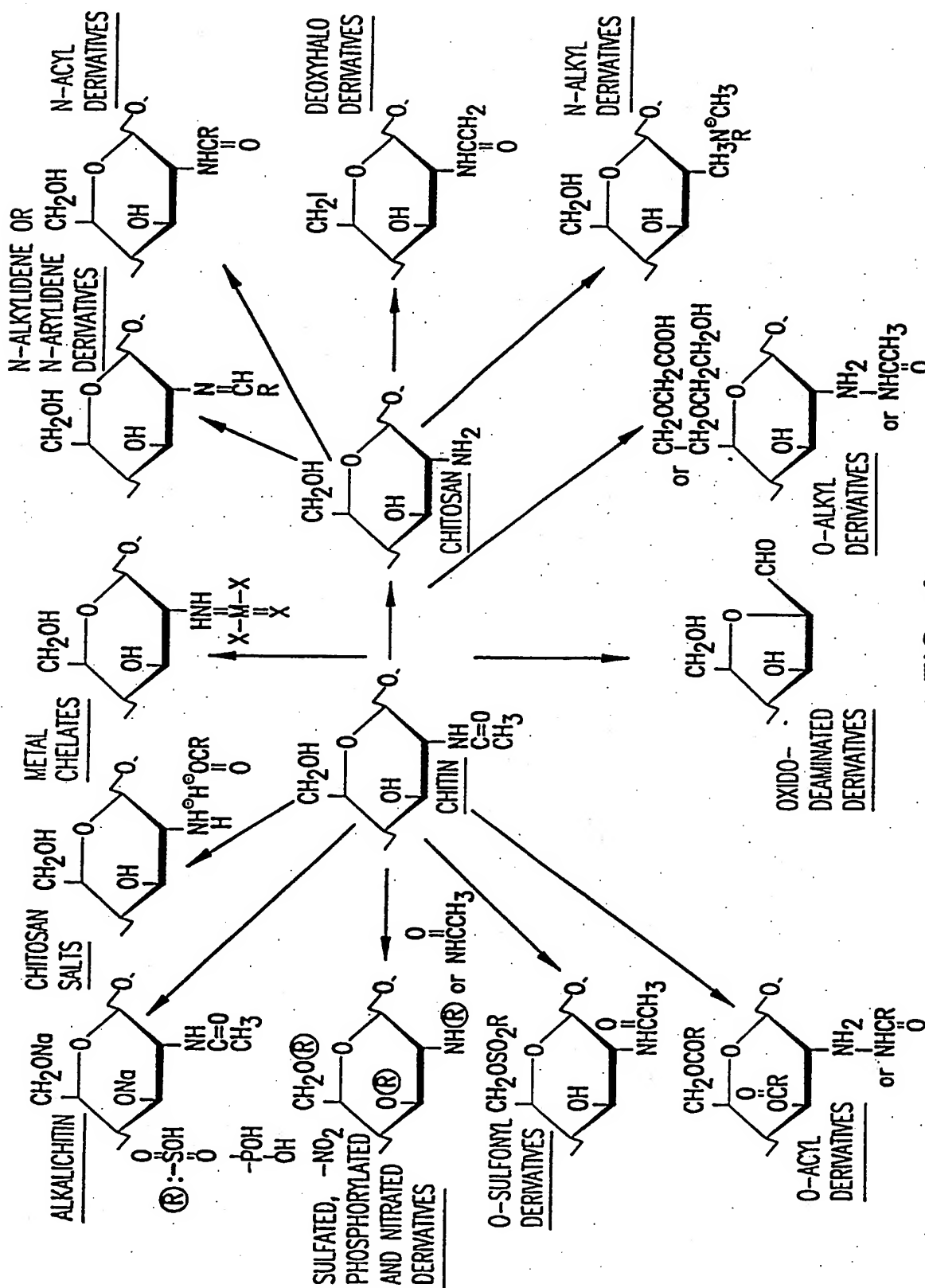


FIG. 4

7867-020

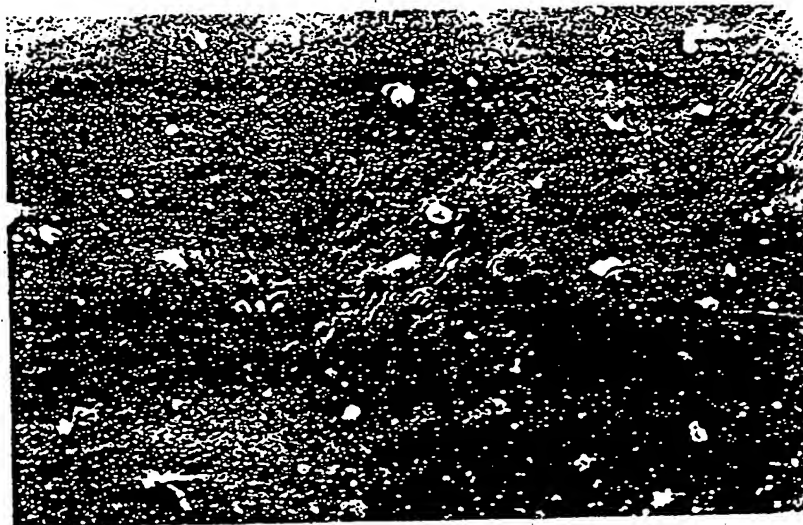


FIG. 5A

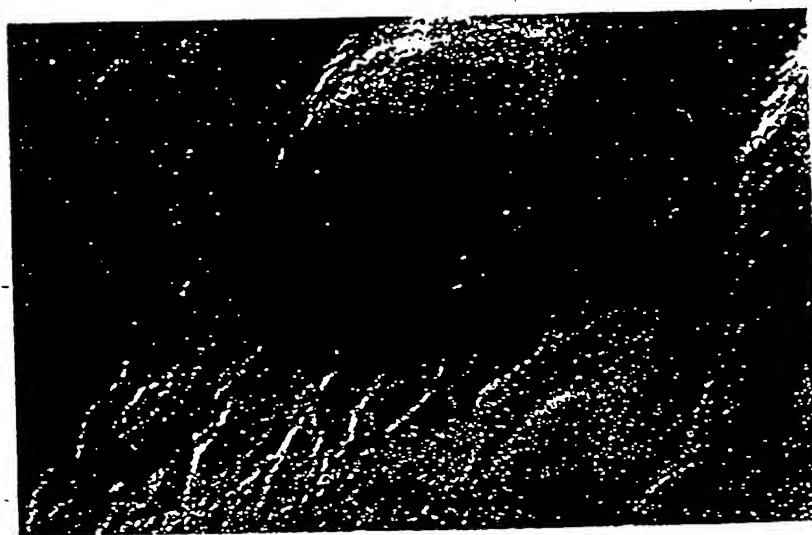


FIG. 5B

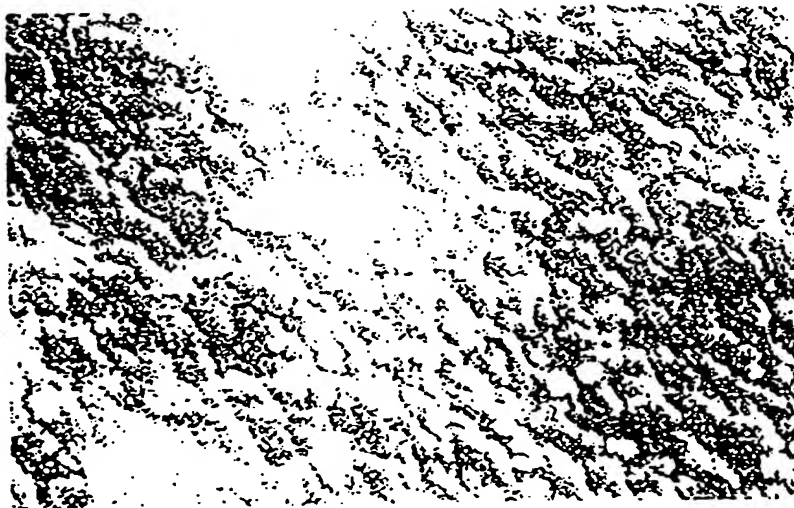


FIG. 6A

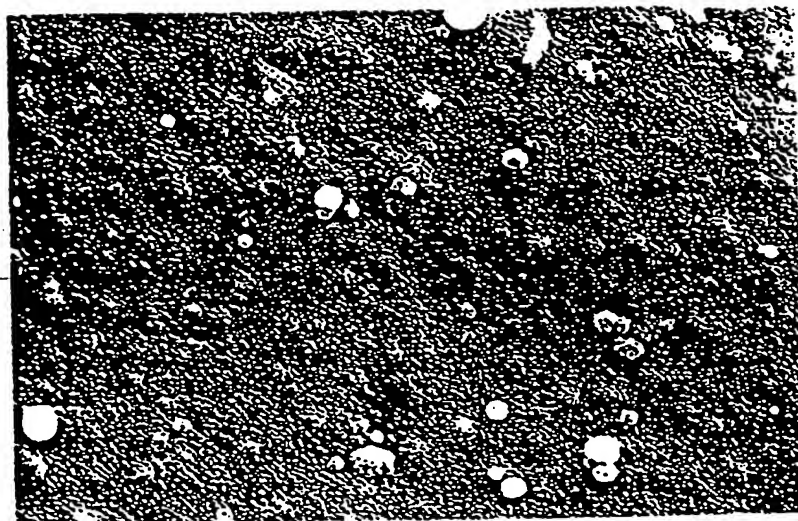


FIG. 6B

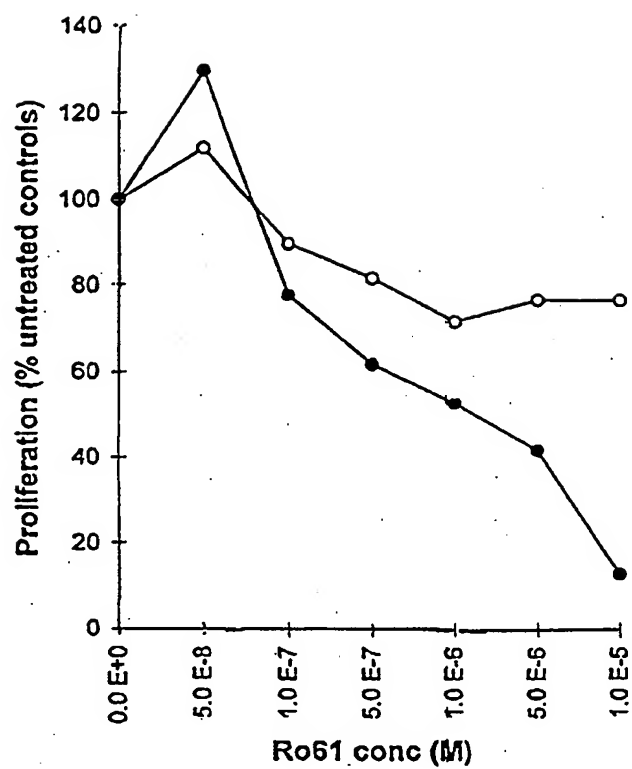


FIG. 7

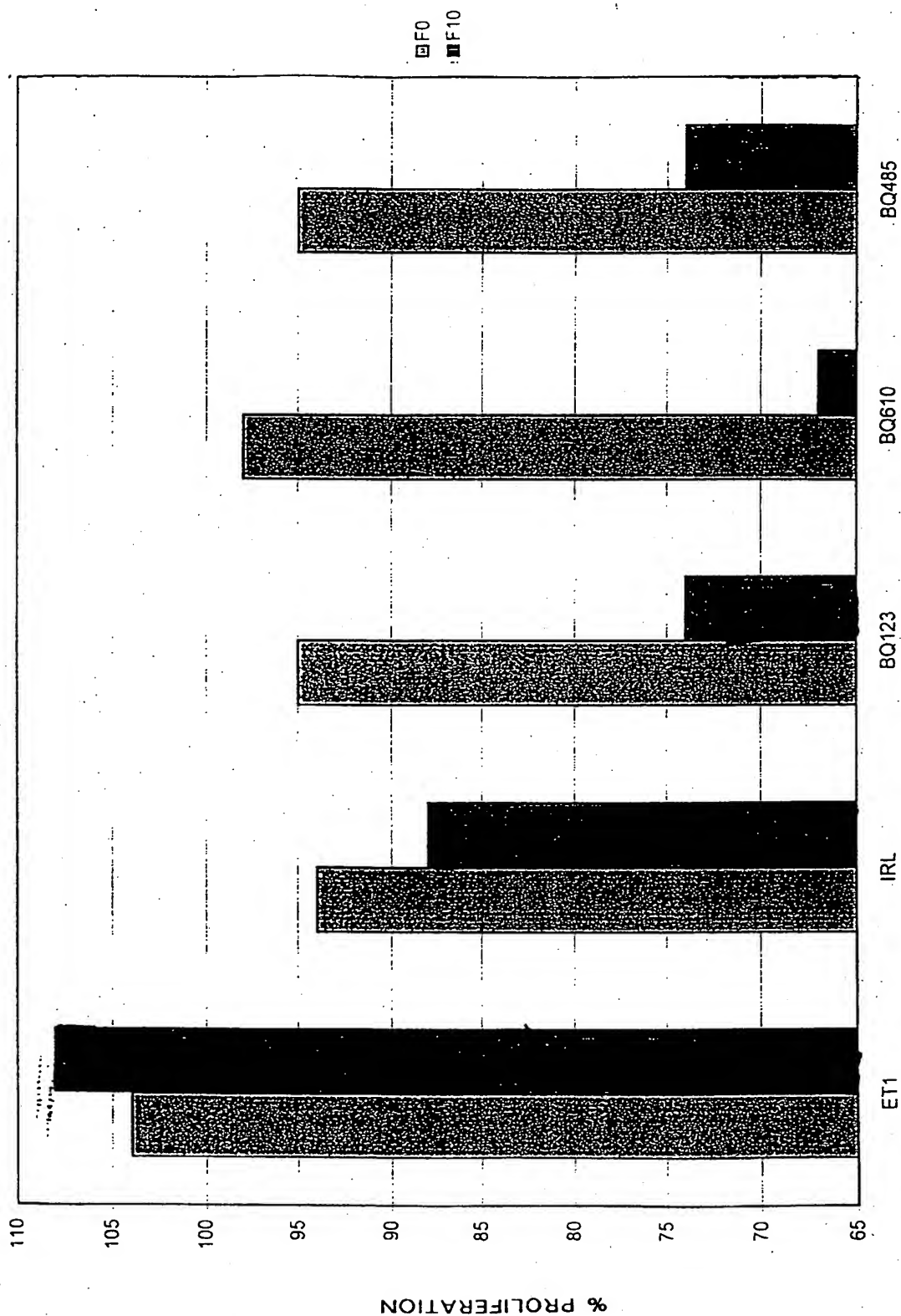


FIG. 8

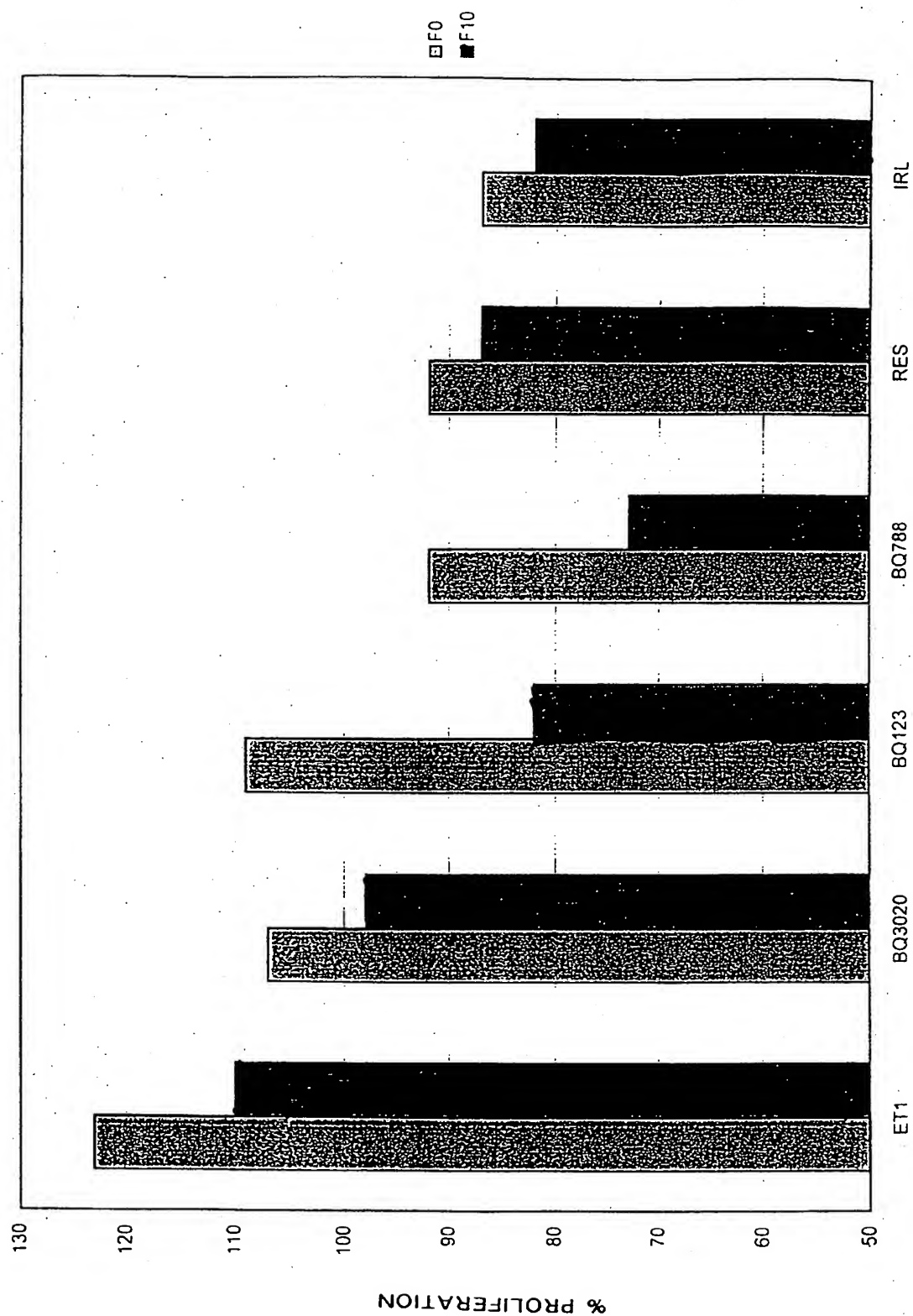


FIG. 9

10 / 17

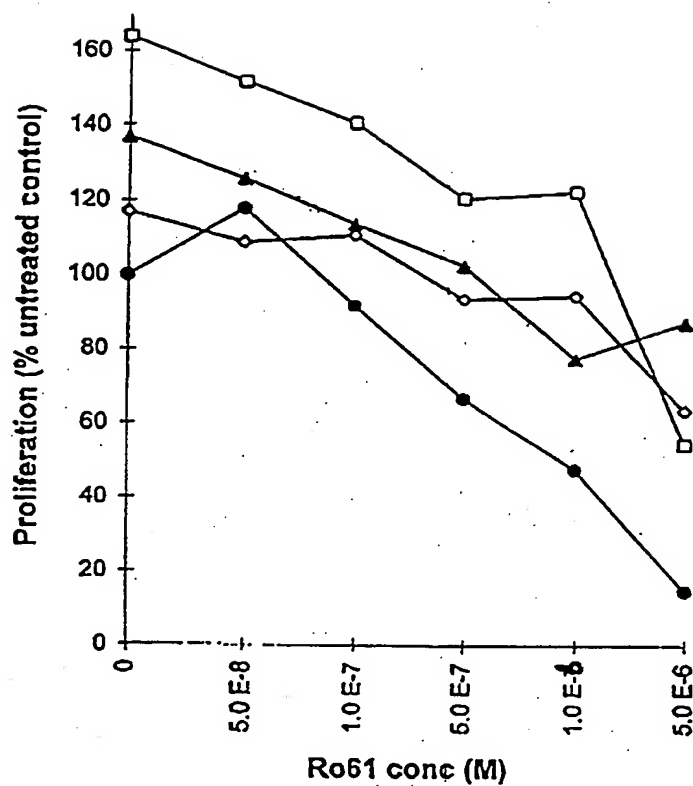


FIG. 10

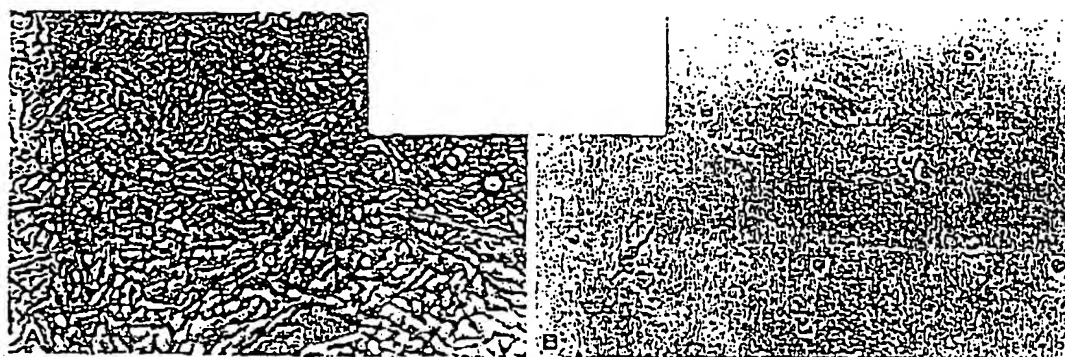


FIG. 11

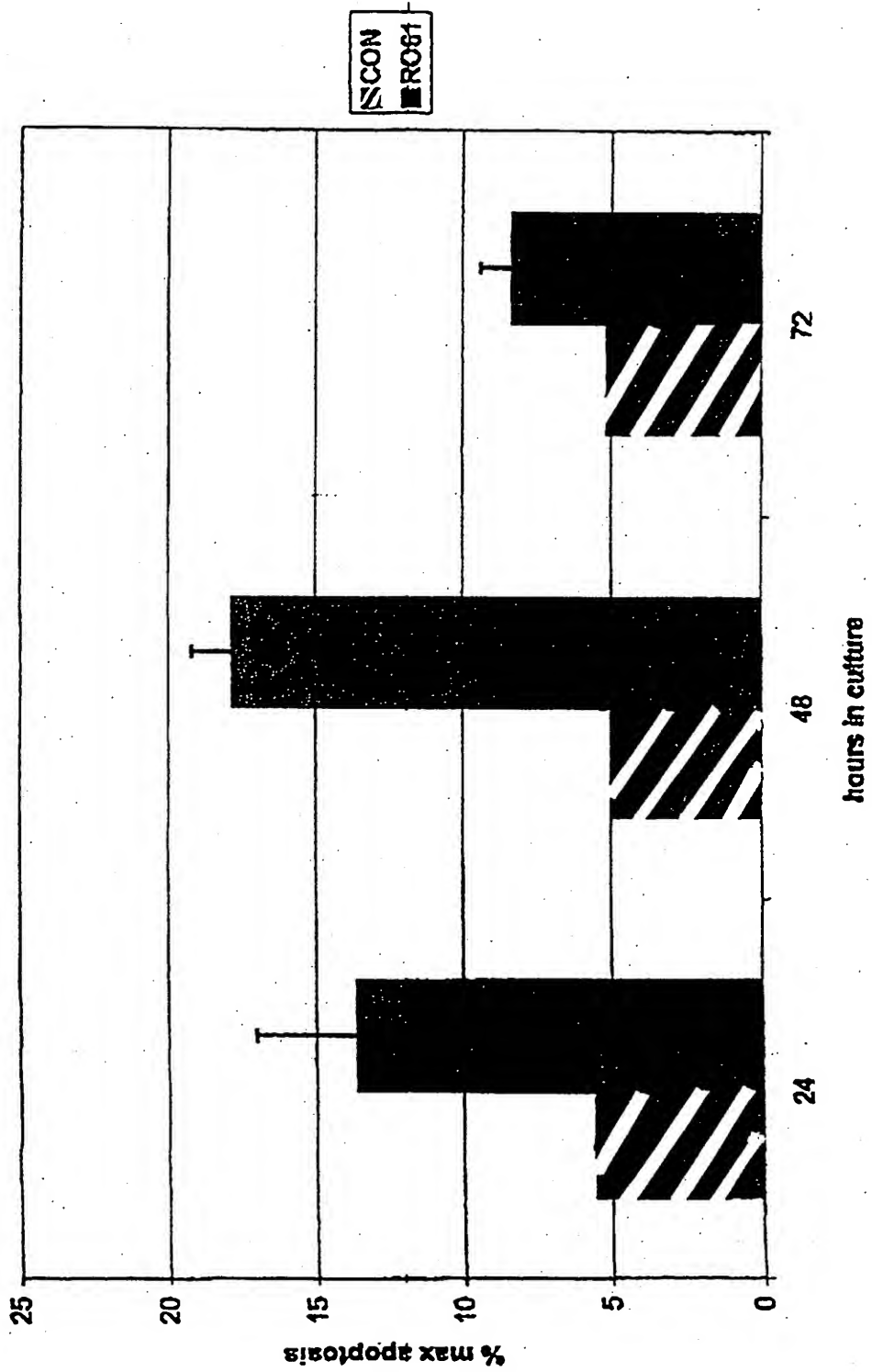


FIG. 12

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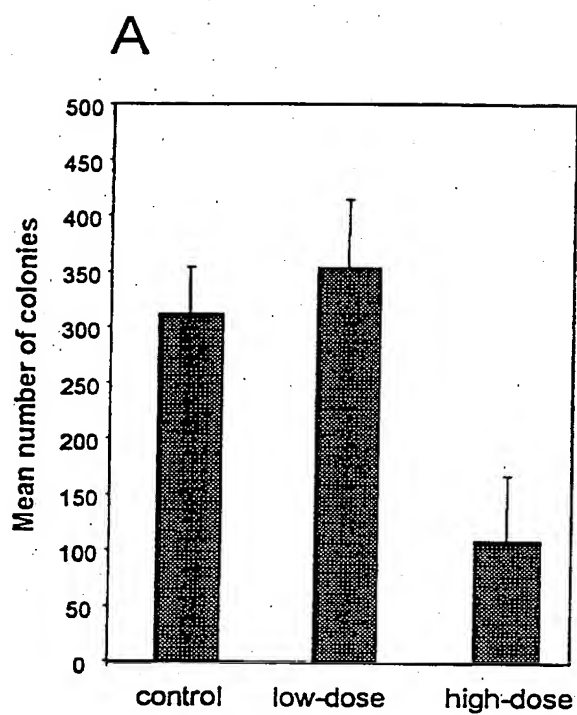


FIG. 13A

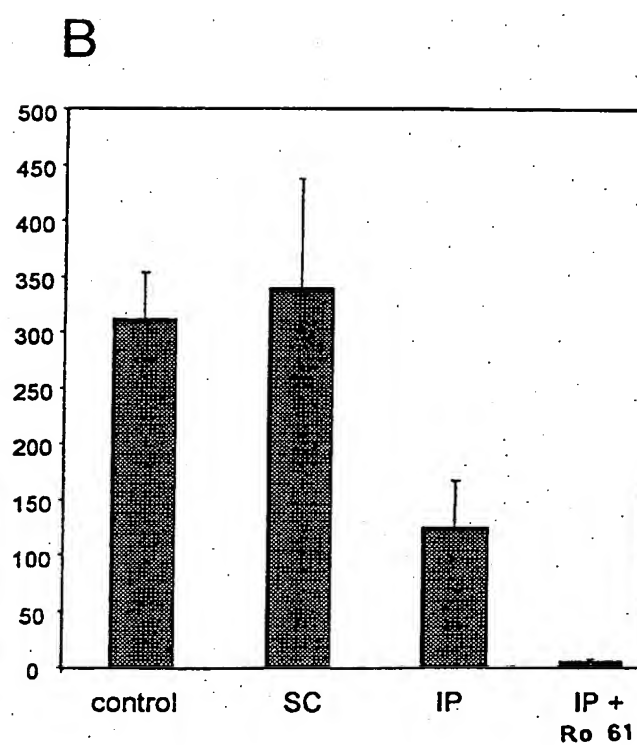


FIG. 13B

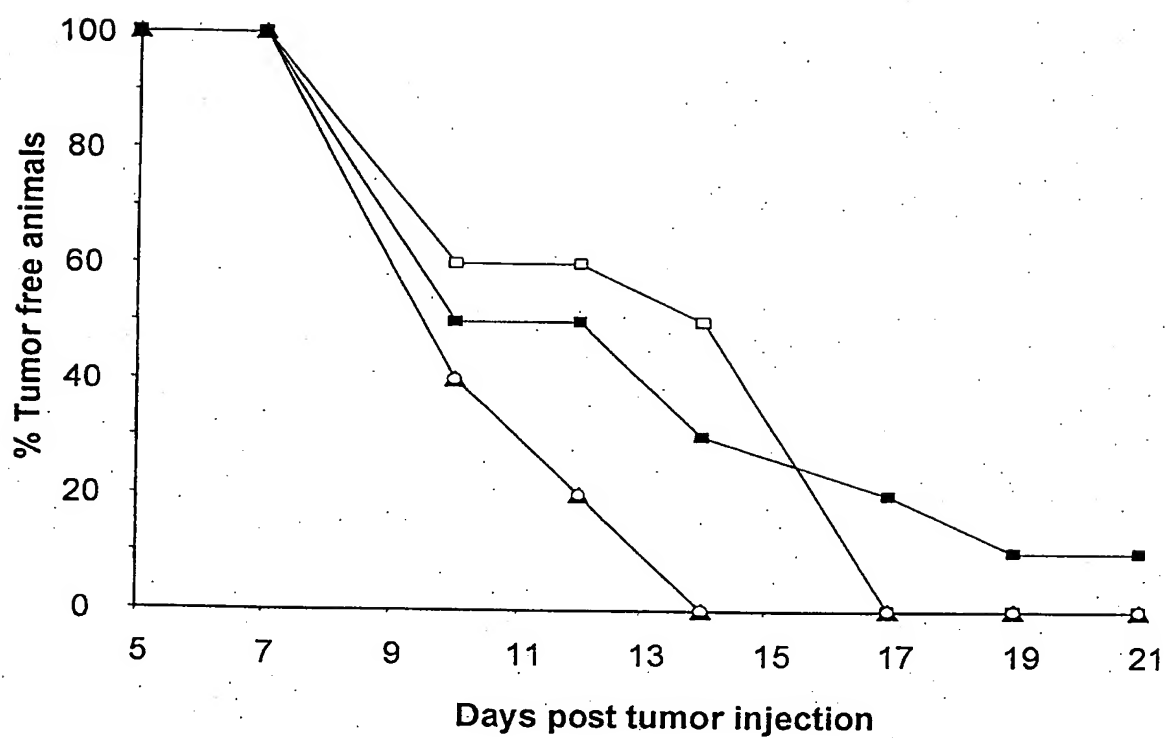


FIG. 14

effect of antagonists on B16 F10 colonies

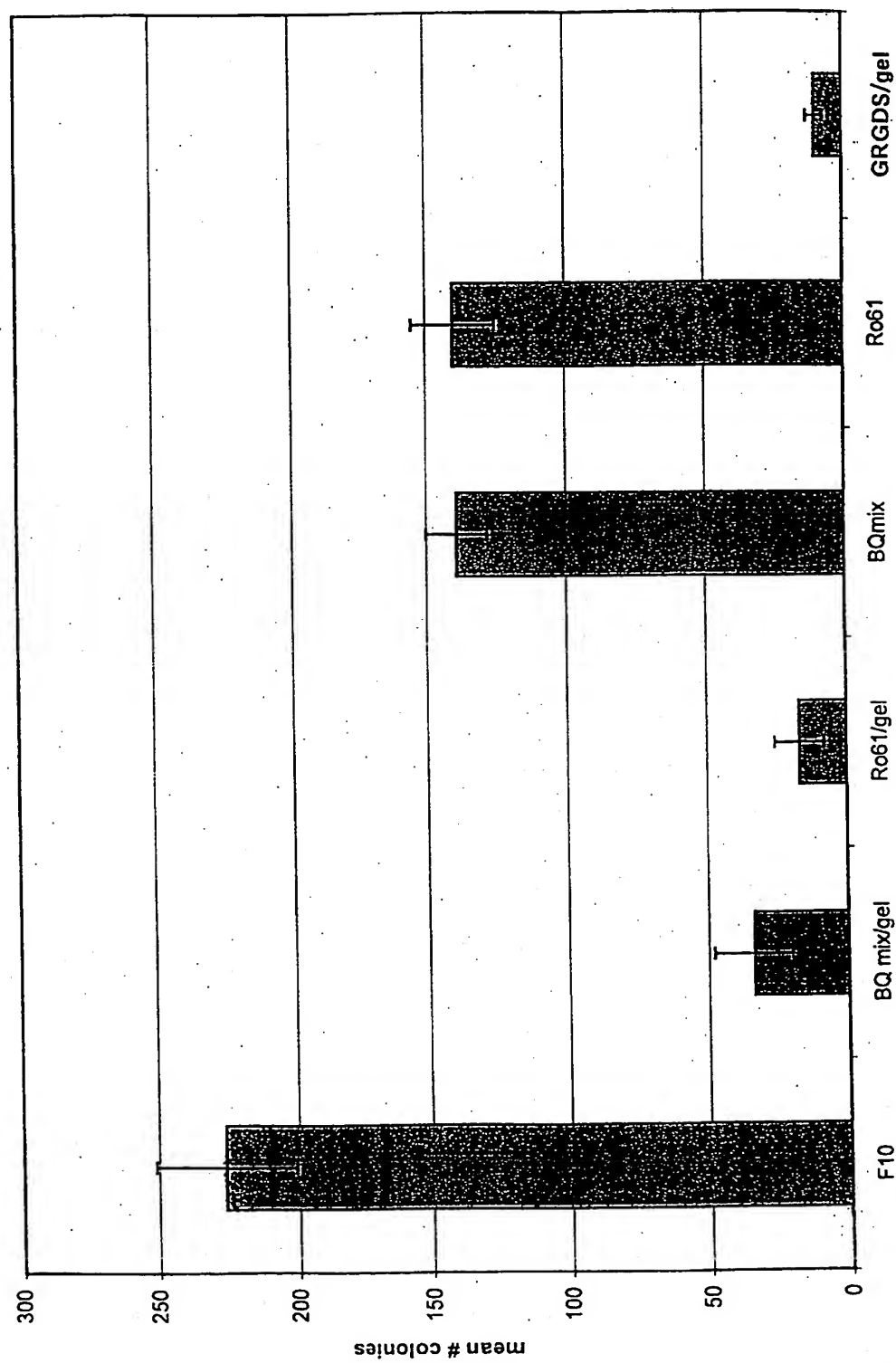


FIG. 15

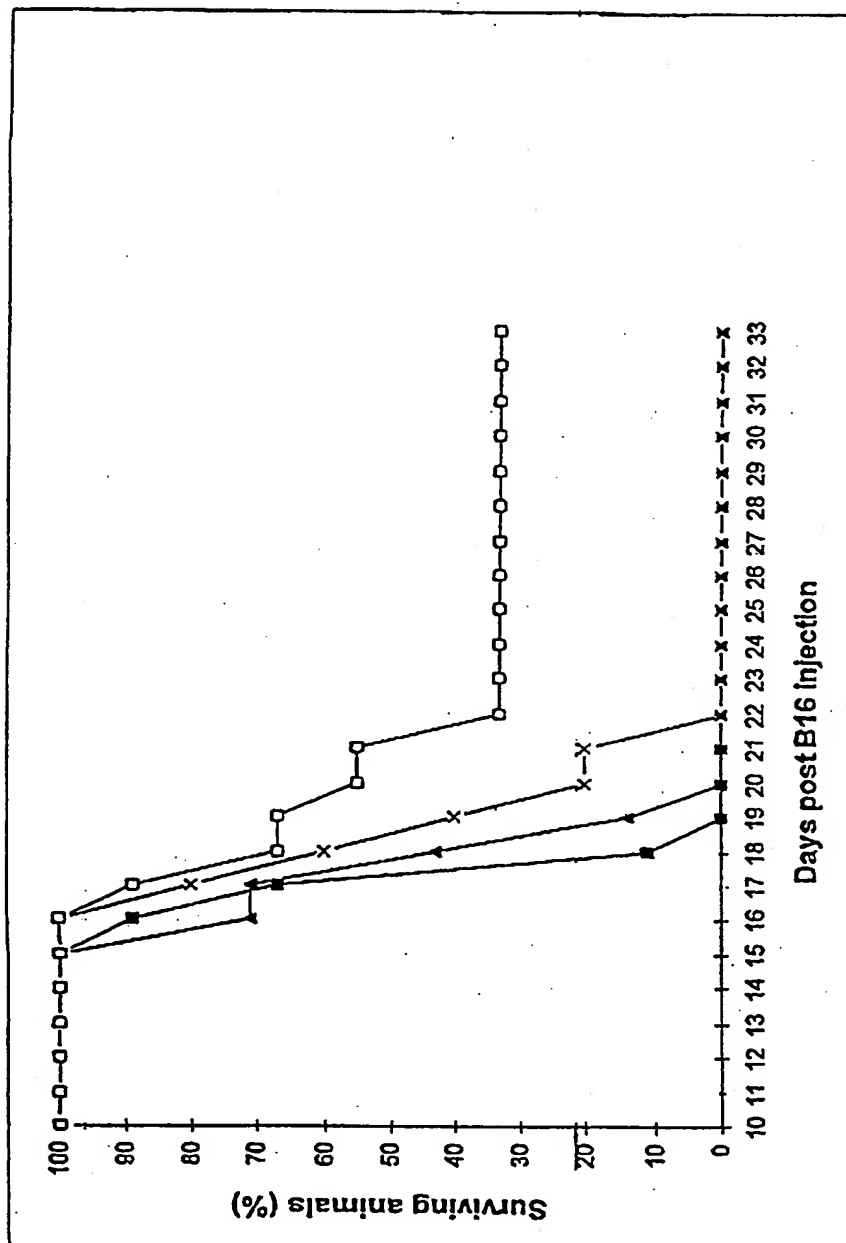


FIG. 16

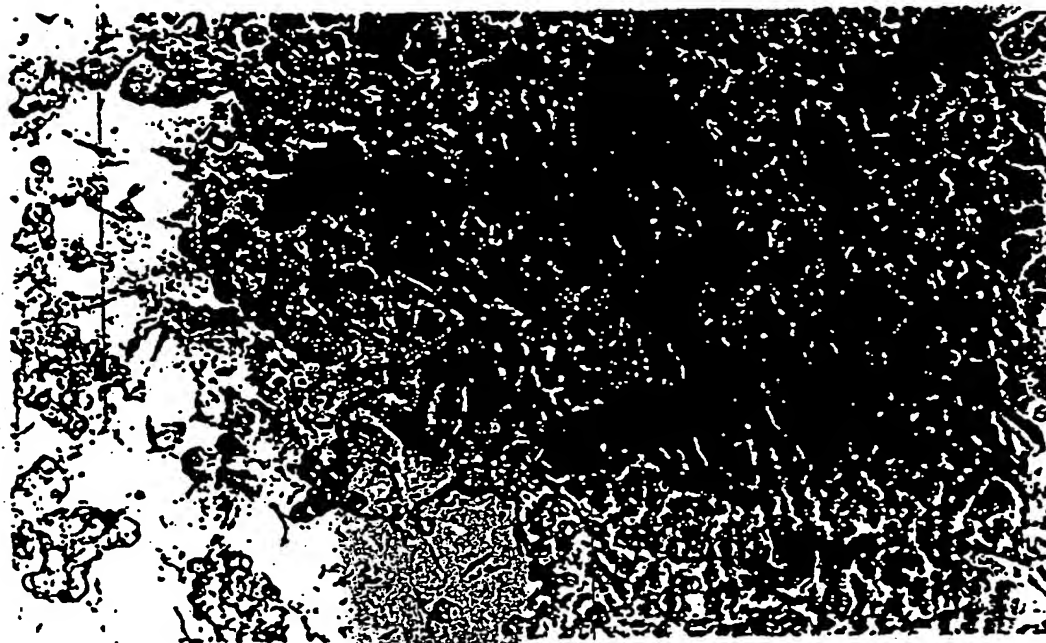


FIG.
17A

B16 10^5 cells RO 10^{-7} M

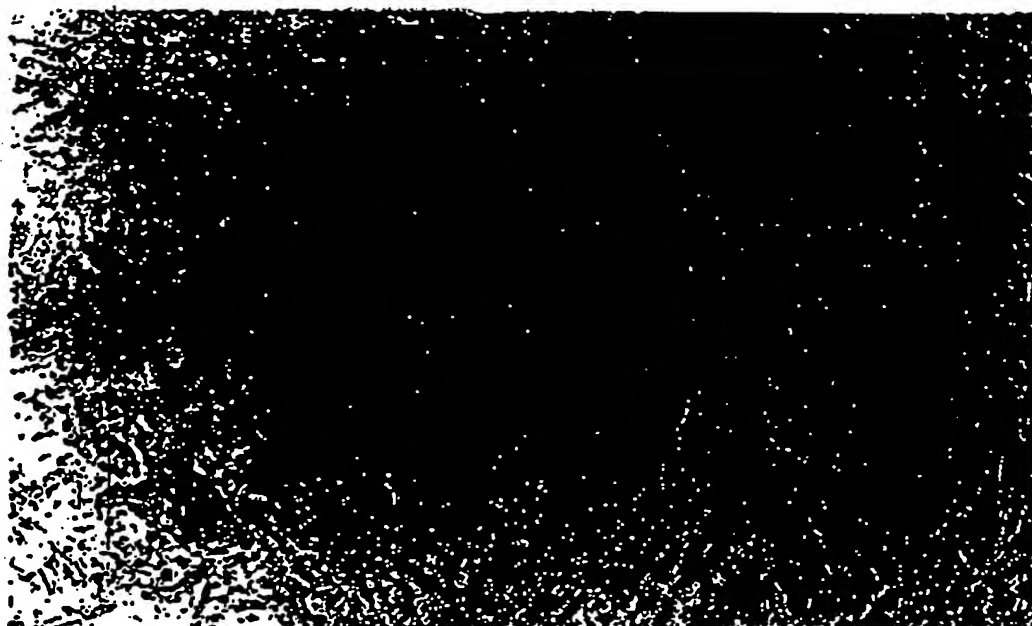


FIG.
17B

B16 10^5 cells no RO

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/30575

A. CLASSIFICATION OF SUBJECT MATTER IPC(7) : A01N 43/04; C08B 37/08 US CL : 514/55; 536/20, 55.2 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 514/55; 536/20, 55.2 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WEST 1.2, STN, MEDLINE, BIOSIS, CAFBUS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,571,821 A (CHAN et al) 05 November 1996 (05.11.96), see entire document, especially abstract and claims.	31-33
X	US 5,541,186 A (BREU et al) 30 July 1996 (30.07.96), see entire document, especially abstract and claims.	31-33
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
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L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document combined with one or more other such documents, such combination being obvious to a person skilled in the art
O	document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
P	document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search 14 MARCH 2000		Date of mailing of the international search report 25 APR 2000
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